Express Mail No. **EV577672616US**

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| **APPELLANT'S BRIEF** | Attorney Docket Confirmation No. | UCAL-161 DIV 7273 |
| | First Named Inventor | S. Finkbeiner |
| | Application Number | 09/922,483 |
| | Filing Date | August 2, 2001 |
| | Group Art Unit | 1648 |
| Address to: Mail Stop Appeal Brief-Patents Commissioner for Patents P.O. Box 1450 Alexandria, VA 22313-1450 | Examiner Name | U. Winkler |
| | Title | *Antibodies specific for proteins having polyglutamine expansions* |

Sir:

This Brief is filed in support of Appellant's appeal from the Examiner's Rejection dated April 19, 2004. No claims have been allowed; and claims 10-13 and 28-30 are pending. Claims 10-13 and 28-30 are appealed. A Notice of Appeal was filed on October 15, 2004. A petition and fee for a four-month extension of time is filed herewith. Accordingly, this Appeal Brief is timely filed.

The Board of Appeals and Interferences has jurisdiction over this appeal pursuant to 35 U.S.C. §134.

The Commissioner is hereby authorized to charge deposit account number 50-0815 in the amount of $250.00 to cover the fee required under 37 C.F.R. §41.20(b)(2) for filing Appellant's brief. In the unlikely event that the fee transmittal or other papers are separated from this document and/or other fees or relief are required, appellants petition for such relief, including extensions of time, and authorize the Commissioner to charge any fees under 37 C.F.R. §§ 41.20(b)(2), 1.16, 1.17 and 1.21 which may be required by this paper, or to credit any overpayment, to Deposit Account No. 50-0815, order number UCAL-161 DIV.

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**REAL PARTY IN INTEREST (i)**

The inventor named on this patent application assigned his entire rights in the invention to The Regents of the University of California. The real party in interest in this application is The Regents of the University of California.

**RELATED APPEALS AND INTERFERENCES (ii)**

There are currently no other appeals or interferences known to Appellant, the undersigned Appellant's representative, or the assignee to whom the inventor assigned his rights in the instant case, which would directly affect or be directly affected by, or have a bearing on the Board's decision in the instant appeal.

**STATUS OF THE CLAIMS (iii)**

Claims 10-13 and 28-30 are pending and are appealed. Claims 1-9 and 14-27 are canceled.

This application was filed on August 2, 2001, and claims benefit of priority to U.S. Patent Application No. 09/478,960, filed January 7, 2000 (now U.S. Patent No. 6,291,652). A Preliminary Amendment was filed along with the application on August 2, 2001, canceling claims 1-9 and 25-27. As a result, claims 10-24 were pending. A Restriction Requirement issued on February 4, 2003; in response to the Restriction Requirement, Group I (claims 10-13) was elected. As a result, claims 14-24 were withdrawn from consideration. In an amendment, filed on October 20, 2003 and responsive to the May 20, 2003 Office Action, claim 10 was amended; and claims 28-30 were added. The October 20, 2003 amendments were entered. In an amendment, filed on July 16, 2004 and responsive to the July 19, 2004 final Office Action, claims 14-24 were canceled. An Advisory Action mailed August 12, 2004 indicated that the July 16, 2004 amendments were entered. As a result of the amendments discussed above, claims 10-13 and 28-30 remain pending.

All of the pending claims 10-13 and 28-30 shown in the Claims Appendix remain pending, rejected, and appealed here.

**STATUS OF AMENDMENTS (iv)**

During the course of prosecution, the above-noted amendments were made. As noted above, an Advisory Action mailed August 12, 2004 indicated that the July 16, 2004 amendments were entered. Thus, all claim amendments have been entered. As a result of the amendments discussed above, claims 10-13 and 28-30 remain pending.

As such, there are no outstanding claim amendments; and claims 10-13 and 28-30 as shown in the Claims Appendix are the claims of record, are pending, and are appealed here.

**SUMMARY OF THE CLAIMED SUBJECT MATTER (v)**

The instant invention provides antibodies, and binding fragments and mimetics of such antibodies, which specifically bind to polyglutamine expansion-containing proteins, such as mutant huntingtin protein. Specification, page 3, lines 23-25; page 4, line 20 to page 6, line 26; and page 8, line 25 to page 9, line 11. The antibodies are useful in screening methods, for identifying compounds for polyglutamine expansion protein binding modulation activity. Specification, page 3, lines 14-16; and page 11, line 24 to page 12, line 30. Diseases such as Huntingtons' Disease (HD) are characterized by the presence of trinucleotide repeat expansions, such as polyglutamine expansions. Specification, page 1, line 23 to page 2, line 18. Agents that modulate binding of a polyglutamine expansion protein and to another protein, e.g., a cellular target of the polyglutamine expansion protein, are expected to be useful in the treatment of diseases such as HD. Specification, page 13, lines 19-22.

The parent application, 09/478,960, issued as U.S. Patent No. 6,291,652, and claims a monoclonal antibody that recognizes a protein having a polyglutamine expansion; a monoclonal antibody that binds to mutant huntingtin protein having a polyglutamine expansion; and binding fragments of the antibodies. The instant case claims screening methods using antibodies that recognize a protein having a polyglutamine expansion to identify compounds that modulate the binding interaction between a polyglutamine expansion-containing protein and a cellular target of the protein (independent claim 10). Specification, page 11, line 24 to page 12, line 30.

**GROUND OF REJECTION TO BE REVIEWED ON APPEAL (vi)**

There is one issue on appeal, as follows:

I. WHETHER CLAIMS 10-13 AND 28-30 MEET THE ENABLEMENT REQUIREMENT OF 35 U.S.C. §112, FIRST PARAGRAPH

**ARGUMENT (vii)**

The arguments portion of this Brief is divided into two sections. The first section describes Appellant's understanding of the Examiner's rejections. The second section specifically addresses the issue outlined above relating to whether the pending claims meet the enablement requirement of 35 U.S.C.§112, first paragraph.

With respect to the rejection under 35 U.S.C.§112, first paragraph as set forth in April 19, 2004 final Office Action, claims 10-13 and 28-30 are argued as a group and stand or fall together.

**Rejection of claims 10-13 and 28-30 under 35 U.S.C. §112, first paragraph**

Claims 10-13 and 28-30 were rejected under 35 U.S.C.§112, first paragraph, as allegedly lacking enablement. In support of this rejection, the Office argued that there is "no correlation in the prior art or the instant specification which would indicate that a compound that interferes with the antibody binding to the polyglutamine expansion of Huntington [sic] would interfere with the binding of the polyglutamine expansion protein to the normal cellular target." Final Office Action, page 5.

The rejection of claims 10-13 and 28-30 under 35 U.S.C.§112, first paragraph, is in error.

*Comments regarding the enablement requirement of 35 U.S.C.§112, first paragraph*

The enablement requirement of 35 U.S.C.§112, first paragraph, requires that the specification describe how to make and how to use the claimed invention. Any analysis of whether a particular claim is supported by the disclosure in an application requires a determination of whether that disclosure, when filed, contained sufficient information regarding the subject matter of the claims as to enable one skilled in the

pertinent art to make and use the claimed invention. The claimed invention must be enabled so that any person skilled in the art can make and use the claimed invention without undue experimentation. *In re Wands*, 858 F.2d 731, 737, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988). The test of enablement is whether one reasonably skilled in the art could make or use the invention from the disclosures in the patent, coupled with information known in the art, without undue experimentation. *United States v. Telectronics, Inc.*, 857 F.2d 778, 785, 8 USPQ2d 1217, 1223 (Fed. Cir. 1988). Determining enablement is a question of law based on underlying factual findings. *In re Vaeck*, 947 F.2d 488, 495, 20 USPQ2d 1438, 1444 (Fed. Cir. 1991); *Atlas Powder Co. v. E.I. du Pont de Nemours & Co.*, 750 F.2d 1569, 1576, 224 USPQ 409, 413 (Fed. Cir. 1984).

In order to make a rejection, the examiner has the initial burden to establish a reasonable basis to question the enablement provided for the claimed invention. *In re Wright*, 999 F.2d 1557, 1562, 27 USPQ2d 1510, 1513 (Fed. Cir. 1993). A specification disclosure which contains a teaching of the manner and process of making and using an invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented must be taken as being in compliance with the enablement requirement of 35 U.S.C.§112, first paragraph, unless there is a reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support. It is incumbent upon the Patent Office, whenever a rejection on the basis of lack of enablement is made, to explain why it doubts the truth or accuracy of any statement in a supporting disclosure and to back up assertions of its own with acceptable evidence or reasoning which is inconsistent with the contested statement. *In re Marzocchi*, 439 F.2d 220, 224, 169 USPQ 367, 370 (CCPA 1971).

If the examiner has weighed all the evidence and established a reasonable basis to question the enablement provided for the claimed invention, the burden falls on applicant to present persuasive arguments, supported by suitable proofs where necessary, that one skilled in the art would be able to make and use the claimed invention using the application as a guide. *In re Brandstadter*, 484 F.2d 1395, 1406-07, 179 USPQ 286, 294 (CCPA 1973). The evidence provided by applicant need not be conclusive but merely convincing to one skilled in the art.

*Comments regarding the claimed invention*

The instant invention as claimed recites a method for determining whether an agent is capable of modulating the binding interaction between the polyglutamine expansion of a protein comprising a polyglutamine expansion (a "polyglutamine expansion-containing protein") and a cellular target of the polyglutamine expansion-containing protein. All that claim 10 requires is that the polyglutamine expansion-containing protein be contacted with the agent and an antibody that recognizes the polyglutamine expansion of the polyglutamine expansion-containing protein (where the antibody has greater affinity for the polyglutamine expansion than a 1C2 monoclonal antibody); and that the presence of a binding complex between the antibody and the polyglutamine expansion-containing protein be detected and compared to a control. The ability of the agent to modulate the binding interaction between the polyglutamine expansion-containing protein and the antibody indicates the ability of the agent to modulate binding interaction between the polyglutamine expansion-containing protein and a cellular target of the polyglutamine expansion-containing protein. Thus, the antibody in the claimed method serves as a surrogate for the cellular target(s) of the polyglutamine expansion-containing protein.

The instant specification provides ample description of antibodies to be used in a subject method; as well as ample description as to how to determine whether an agent modulated binding of an antibody to a polyglutamine expansion of a polyglutamine expansion-containing protein. The specification describes antibodies, and binding fragments and mimetics of such antibodies, which specifically bind to polyglutamine expansion-containing proteins, such as mutant huntingtin protein. Specification, page 3, lines 23-25; page 4, line 20 to page 6, line 26; and page 8, line 25 to page 9, line 11; and page 18, line 28-page 19, line 1. The specification describes how to determine whether an agent modulated binding of an antibody to a polyglutamine expansion of a polyglutamine expansion-containing protein. Specification, page 11, line 24 to page 12, line 30. Given the description in the specification, those skilled in the art could readily practice the method as claimed without undue experimentation.

Antibodies are proteins that bind to antigens, which can include protein antigens. The person skilled in the art would find it reasonable that cellular target(s) for polyglutamine expansion-containing protein include proteins. The person skilled in the art would find it reasonable that a polyglutamine expansion-containing protein binds to an antibody specific for the polyglutamine expansion of the polyglutamine

expansion-containing protein in a manner similar to the binding of a cellular target of the polyglutamine expansion-containing protein. The person skilled in the art would also find it reasonable to use an antibody as a surrogate for the cellular target(s) of a polyglutamine expansion-containing protein in a screening method to identify agents that modulate binding interaction between a polyglutamine expansion-containing protein and a cellular target of the protein.

*The Examiner has failed to establish a reasonable basis to question the enablement provided for the claimed invention.*

The April 19, 2004 Office Action stated that there is "no correlation in the prior art or the instant specification which would indicate that a compound that interferes with the antibody binding to the polyglutamine expansion of Huntington [sic] would interfere with the binding of the polyglutamine expansion protein to the normal cellular target." April 19, 2004 Office Action, page 5. However, the instant specification asserts that an antibody that binds a polyglutamine expansion of a polyglutamine expansion-containing protein can be used to identify agents that modulate the binding interaction between a polyglutamine expansion-containing protein and a cellular target of the protein. Specification, page 11, lines 24-29. It is incumbent upon the Patent Office to explain why it doubts that an antibody that binds a polyglutamine expansion of a polyglutamine expansion-containing protein can be used to identify agents that modulate the binding interaction between a polyglutamine expansion-containing protein and a cellular target of the protein; and to back up assertions of its own with acceptable evidence or reasoning.

The April 19, 2004 Office Action further stated that "[m]easuring the interaction between the polyglutamine expansion protein and the antibody **will not provide any insights** between the interaction of the polyglutamine protein binding to its unknown and undisclosed putative cellular protein." April 19, 2004 Office Action, page 5, emphasis added. However, this appears to be a personal opinion of the Examiner and is unsupported by any evidence or scientific reasoning.

The April 19, 2004 Office Action stated that "[a] compound that interferes with the antibody binding to the polyglutamine expansion protein can act on the antibody alone or it can bind to the polyglutamine expansion protein" and further stated that "the instantly claimed method cannot determine to which protein the agent binds, therefore, the claimed method cannot determine if the agent is capable of modulating the

interaction between the polyglutamine expansion protein and the cellular target." April 19, 2004 Office Action, page 5. However, whether the agent binds to the antibody or to the polyglutamine expansion of the polyglutamine expansion-containing protein is irrelevant in terms of protein-protein binding. Many agents are known that modulate protein-protein binding between two different proteins. Whether the agent binds to one protein or the other to modulate the binding is irrelevant. What is relevant is that the binding is modulated.

The May 20, 2003 Office Action stated that Heiser et al. ((2000) *Proc. Natl. Acad. Sci. USA* 97:6739-6744; "Heiser") teaches that the monoclonal antibody (MAb) 1C2 specifically recognizes polyglutamine expansions in soluble huntingtin, and that the MAb does not recognize insoluble high molecular weight polyglutamine expansions. The May 20, 2003 Office Action stated that Heiser teaches a screening assay that interferes with the self aggregation of huntingtin; and that Heiser indicates that the 1C2 monoclonal antibody, the HD1 polyclonal antibody, as well as Congo Red are able to prevent the huntingtin protein from aggregating. The May 20, 2003 Office Action stated that there is no correlation in the prior art or the instant specification which would indicate that a compound that interferes with the antibody binding to the polyglutamine expansion of huntingtin would interfere with the binding of the polyglutamine expansion protein to the normal cellular target.

However, no conclusion as to the enablement of the instant claims can be drawn from the disclosure of Heiser. As the May 20, 2003 Office Action acknowledged, Heiser examined interference with self aggregation of huntingtin. Heiser does not disclose agents that inhibit a binding interaction between an antibody to a polyglutamine expansion of a polyglutamine expansion-containing protein; and does not comment on the relevance of same to the identification of agents that modulate binding interaction between a polyglutamine expansion-containing protein and a cellular target of the protein.

*Antibody specific for a protein can serve as a surrogate for binding of that protein to a second protein*

Illustrative of the principle that an antibody that is specific for a protein can serve as a surrogate, or a model, for the binding interaction between that protein and a second protein are the following references:

Kaji et al. ((2001) *J. Biochem.* 129:577-583; "Kaji"); and South et al. ((1995) *Thromb. Haemost.* 73:144-150; "South"). Copies of these references were provided as Exhibits 1 and 2, respectively, along with the amendment, filed on October 20, 2003 and responsive to the May 20, 2003 Office Action. Copies of these references are provided herewith as a courtesy.

Kaji

Kaji discusses screening a phage display library with monoclonal antibodies that inhibit the chemotactic activity of monocyte chemoattractant protein-1 (MCP-1). Phage clones that were bound by the antibodies were isolated and characterized. Two peptides were identified that bound to THP-1 cells (which are responsive to MCP-1); the binding was competitively inhibited by MCP-1. Kaji concluded that the peptides mimic the MCP-1 binding domain that is recognized by the MCP-1 receptor. Thus, Kaji successfully used antibody binding to a domain on the MCP-1 protein as a surrogate, or a model, for the binding of the MCP-1 protein to its cellular target, i.e., the MCP-1 receptor. Using the antibody as a surrogate for the MCP-1 receptor, Kaji found peptides that bound the antibody, and that also bound the MCP-1 receptor. In a manner analogous to Kaji, the instant claims use antibody specific for a polyglutamine expansion of a polyglutamine expansion-containing protein to identify agents that modulate the binding interaction between the polyglutamine expansion-containing protein and its cellular target.

The April 19, 2004 Office Action stated that Kaji differs from the instant specification in that Kaji associated an activity that is inhibited with the antibody. However, as noted above, the instant specification asserts that an antibody that binds a polyglutamine expansion of a polyglutamine expansion-containing protein can be used to identify agents that modulate the binding interaction between a polyglutamine expansion-containing protein and a cellular target of the protein. Specification, page 11, lines 24-29. The Examiner has not provided acceptable evidence or reasoning to explain why she doubts that an antibody that binds a polyglutamine expansion of a polyglutamine expansion-containing protein can be used to identify agents that modulate the binding interaction between a polyglutamine expansion-containing protein and a cellular target of the protein.

The April 19, 2004 Office Action merely stated that "[t]he specification has not provided any

evidence that the antibodies prevent the binding of the Huntington protein to the unknown and undisclosed putative cellular receptor." April 19, 2004 Office Action, page 3. The Examiner has apparently misunderstood the claimed invention. The antibodies in the claimed method do not serve to prevent the binding of the polyglutamine expansion-containing protein to its cellular target. Instead, the antibodies serve as surrogates for the cellular target.

South

South discusses screening a phage display library for inhibitors of the von Willebrand factor (vWF)-platelet Glycoprotein Ib (GPIb) interaction. The phage display library was screened with a monoclonal antibody that recognizes the GPIb binding domain of vWF. Phage clones were identified that reacted with the monoclonal antibody. A number of the peptides thus identified inhibited binding of vWF to GPIb. Thus, South successfully used antibody binding to a domain of vWF as a surrogate, or a model, for vWF-GPIb binding; and, using the monoclonal antibodies, successfully identified peptides that inhibited vWF-GPIb binding. In a manner analogous to South, the instant claims use antibody specific for a polyglutamine expansion of a polyglutamine expansion-containing protein to identify agents that modulate the binding interaction between the polyglutamine expansion-containing protein and its cellular target.

The April 19, 2004 Office Action stated that South differs from the instant specification in that the authors of South knew that the antibody binds to the GPIb domain that recognizes vWF. The April 19, 2004 Office Action further stated that in the instant specification there is no correlation provided that the region to which the antibody binds is the same region that is responsible for the binding of the polyglutamine expansion-containing protein to the normal cellular receptor. However, as noted above, those skilled in the art would find it reasonable that a polyglutamine expansion-containing protein binds to an antibody specific for the polyglutamine expansion of the polyglutamine expansion-containing protein in a manner similar to the binding of a cellular target of the polyglutamine expansion-containing protein. Therefore, those skilled in the art would also find it reasonable to use an antibody as a surrogate for the cellular target(s) of a polyglutamine expansion-containing protein in a screening method to identify agents that modulate binding interaction between a polyglutamine expansion-containing protein and a cellular target of the protein. The

Examiner has not provided sufficient reasoning as to why those skilled in the art would not find it reasonable to use an antibody to the polyglutamine expansion in the claimed method, to identify agents capable of modulating the binding interaction between a polyglutamine expansion-containing protein and a cellular target of the protein.

*Those skilled in the art would find it reasonable to use a claimed assay method to identify agents that inhibit binding of a protein containing a polyglutamine expansion to its cellular target.*

As noted above, the Examiner has not established a reasonable basis to question the enablement provided for the claimed invention. Even if the Examiner had established a reasonable basis to question the enablement provided for the claimed invention, and it is the Appellant's position that the Examiner has not done so, evidence has been provided that those skilled in the art would find it convincing that the instant specification is enabling for the claimed invention.

A Declaration of Ross Stein (the "Stein Declaration") was provided along with the amendment, filed on July 16, 2004 and responsive to the April 19, 2004 Office Action. A copy of the Stein Declaration is provided herewith as a courtesy. As noted in the Stein Declaration, Dr. Stein is an expert in the field of enzyme kinetics and drug discovery. The Stein Declaration stated that it is reasonable to use an assay as claimed to identify agents that inhibit binding of a protein containing a polyglutamine expansion to its cellular target, and that such an assay represents an important and valuable approach to identifying new therapeutic agents for neurodegenerative diseases. The Stein Declaration stated that a grant application describing the screening approach was reviewed by a panel, and indicated that the panel was in support of this approach. Indeed, the grant eventually received a 1.4% priority score, meaning that it scored better than 98.6% of other grant. Thus, those skilled in the art would find it convincing that the instant specification is enabling for the claimed invention.

The Advisory Action stated that the Stein Declaration has been entered and considered. The Advisory Action stated that the Stein Declaration "does not actually indicate that the antibody binding to the polyglutamine expansion protein binds in the same place where the cellular receptor binds to the polyglutamine expansion protein." Advisory Action, page 2. However, the Stein Declaration provides

evidence that those skilled in the art would find it convincing that the instant specification is enabling for the claimed invention. As noted above, the evidence provided by applicant need not be conclusive but merely convincing to one skilled in the art. The Stein Declaration provides ample evidence that those skilled in the art would find it convincing that the instant specification is enabling for the claimed invention.

The Advisory Action quoted the Stein Declaration as stating that "[i]t is not unreasonable to assume that the binding site on the antibody might have structural features and binding properties for polyglutamine that are similar to the binding sites on the cellular proteins that mediate the toxic effect of the polyglutamine." The Advisory Action stated that the Stein Declaration "only speculates that that this could be the case but does not necessarily have to be the case." However, as noted above, the evidence provided by applicant need not be conclusive but merely convincing to one skilled in the art. The Stein Declaration provides ample evidence that those skilled in the art would find it convincing that the instant specification is enabling for the claimed invention.

Conclusion as to the rejection under 35 U.S.C.§112, first paragraph

The present invention provides ample description of how to use the claimed invention without undue experimentation. Those skilled in the art would find it reasonable to use the instant method as claimed to identify agents that modulate binding between a polyglutamine expansion-containing protein and a cellular target of the protein. The examiner has not established a reasonable basis to question the enablement provided for the claimed invention. Accordingly, the instant invention as claimed meets the enablement requirement of 35 U.S.C.§112, first paragraph.

Appellants respectfully request that the rejection of claims 10-13 and 28-30 under 35 U.S.C.§112, first paragraph, be reversed, and that the application be remanded to the Examiner with instructions to issue a Notice of Allowance.

Respectfully submitted,
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Date: Apr. 15, 2005

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## CLAIMS APPENDIX (viii)

10. A method of determining whether an agent is capable of modulating the binding interaction between a protein comprising a polyglutamine expansion and a cellular target of said protein, said method comprising:

(a) contacting said protein or a binding fragment or mimetic thereof with:

(i) said agent; and

(ii) an antibody that recognizes a protein comprising a polyglutamine expansion or binding fragment or mimetic thereof, wherein said antibody has greater affinity for said polyglutamine expansion than a 1C2 monoclonal antibody;

(b) detecting the presence of binding complexes comprising said protein and said antibody; and

(c) comparing the results of step (b) with a control;

wherein the ability of an agent to modulate the binding interaction between said protein and said antibody indicates that the ability of said agent to modulate the binding interaction between said protein and a cellular target of said protein.

11. The method according to Claim 10, wherein said antibody is a monoclonal antibody.

12. The method according to Claim 10, wherein said agent is a small molecule.

13. The method according to Claim 10, wherein a plurality of agents are assayed simultaneously.

28. The method according to claim 10, wherein said protein is a mutant huntingtin protein.

29. The method according to claim 10, wherein said antibody is attached to a solid support.

30. The method according to claim 10, wherein said protein having a polyglutamine expansion is attached to a solid support.

## EVIDENCE APPENDIX (ix)

1) Kaji et al. ((2001) *J. Biochem.* 129:577-583;

2) South et al. ((1995) *Thromb. Haemost.* 73:144-150; "South");

3) Declaration of Ross Stein under 37 C.F.R.§1.132

4) Curriculum vitae of Ross Stein

Items (1) and (2) were submitted as Exhibits 1 and 2, respectively, along with the amendment, filed on October 20, 2003 and responsive to the May 20, 2003 Office Action.

Items (3) and (4) were submitted as Exhibits 1 and 2, respectively, along with the amendment, filed on July 16, 2004 and responsive to the April 19, 2004 Office Action.

## RELATED PROCEEDINGS APPENDIX (x)

There are currently no other appeals or interferences known to Appellant, the undersigned Appellant's representative, or the assignee to whom the inventor assigned his rights in the instant case, which would directly affect or be directly affected by, or have a bearing on the Board's decision in the instant appeal.

VOLUME 129 NO.4 APR 2001

Exhibit 1
U.S. Patent Application No. 09/922,483

# THE JOURNAL OF BIOCHEMISTRY.

BIOCHEMISTRY
MOLECULAR BIOLOGY
CELL BIOTECHNOLOGY

2001

Published Monthly by THE JAPANESE BIOCHEMICAL SOCIET

J. Biochem. 129, 577–583 (2001)

# Peptide Mimics of Monocyte Chemoattractant Protein–1 (MCP-1) with an Antagonistic Activity

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Received December 15, 2000; accepted January 18, 2001

**In this study, we attempted to analyze the peptide motifs recognized by 24822.111 and F9, monoclonal antibodies (mAbs) that inhibit the chemotactic activity of monocyte chemoattractant protein–1 (MCP-1), a member of the CC subfamily of chemokines. We isolated phage clones from a phage display library and identified six peptide motifs. One of these clones, C27, was strongly and specifically recognized by 24822.111 mAb, while another, G25, was similarly recognized by F9 mAb. Both the C27 motif and the G25 motif contain two cysteines in their sequences and have little homology to the primary amino acid sequence of MCP-1. These clones, however, bound to THP-1 cells, and the binding was competitively inhibited by MCP-1. The clones strongly inhibited the MCP-1–induced chemotaxis of human monocytes. The synthetic and intramolecularly disulfide-linked peptides of C27 and G25 (sC27 and sG25) also inhibited the chemotaxis induced by MCP-1, while their derivatives with serine in place of cysteine did not, suggesting the importance of the loop structure for the inhibition. These results suggest that sC27 and sG25 may mimic the MCP-1-binding domain to the MCP-1 receptor.**

**Key words: inhibition, MCP-1, molecular design, peptide mimic, phage library.**

Random-peptide-displaying phage library clones selected by a conformational epitope-recognizing and inhibitory monoclonal antibody (mAb) may display moieties that mimic a receptor/ligand-like three-dimensional structure. This pseudoreceptor/ligand should be able to bind to natural ligand/receptor molecules. We have tested this approach employing rather complex structural molecules such as CTLA4, a negative regulator of T cell function, which belongs to the immunoglobulin supergene family molecules (*1, 2*), and a chemokine receptor with seven transmembrane-domains as target molecules (*3*). In these two cases, we demonstrated that the HPLC-purified gene-3 protein (g3p) displaying the selected motif exhibited particularly antagonistic activity, *i.e.*, a strong augmenting activity on the T cell proliferation in the case of CTLA4 (*1, 2*) and an inhibitory activity on the M-tropic HIV-1 infection in the case of CCR5 (*3*). However, in both cases, synthetic peptides of these motifs failed to reproduce the antagonistic activities of the motif-bearing g3ps.

To examine further the feasibility of the method to copy the three-dimensional image of the binding site by the mAb recognizing the binding epitope, we tested the idea using a human chemokine, monocyte chemoattractant protein–1 (MCP-1), to design MCP-1-mimic peptides recognized by anti–MCP-1 mAbs.

MCP-1 was identified from the culture supernatant of the human glioma cell line U105 MG (*4, 5*) and is a 76–amino acid polypeptide belonging to the CC subfamily of the chemokine family based on the adjacent position of the two cysteines. Its main biological activity is its chemotactic activity for monocytes, and the CC chemokine receptor 2 (CCR2) is a high-affinity receptor for MCP-1 (*6–8*). The three-dimensional structure of the MCP-1 homodimer was determined by heteronuclear multidimensional NMR at a full high resolution (*9*). MCP-1 is involved in the development of asthma, glomerulonephritis, rheumatoid arthritis, atherosclerosis, inflammatory bowel disease, and meningitis as well as in the accumulation of macrophages in tumor sites (*10–16*).

In this study, we employed the anti–MCP-1 mAbs 24822.111 and F9, which inhibit the chemotactic activity of MCP-1, and identified six distinct phage clones, each bearing a different motif. We characterized here two types of phage clones, C27 isolated from 24822.111 mAb and G25 isolated from F9 mAb, in terms of their binding ability to the MCP-1 receptors and their inhibitory activity against the MCP-1–induced chemotaxis. Furthermore, we demonstrated that the synthetic peptides of the C27 or the G25 motif (cC27 or sG25) inhibited the MCP-1–induced chemotaxis of human monocytes.

These results suggested that sC27 and sG25 might mimic the MCP-1–binding domain to the MCP-1 receptor and thus supported the usefulness of the molecular design employing a conformation-epitope-recognizing mAb and a phage-display library.

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Abbreviations: PBMC, peripheral blood mononuclear cells; MCP-1, monocyte chemoattractant protein–1; FBS, fetal bovine serum; g3p, gene 3 protein; mAb, monoclonal antibody.

## MATERIALS AND METHODS

*Antibodies, Chemokines, and Synthetic Peptides*—Anti-human MCP-1 mAb 24822.111 and anti-human CCR2 mAb 48607.121 were purchased from R&D Systems, Inc. (Minneapolis, MN). F9 was prepared by T. Yoshimura (*17*). MCP-1 and IL-8 were purchased from Pepro Tech, Inc. (Rocky Hill, NJ). The chemically synthesized synthetic peptides (19mers) of C27 (sC27) and G25 (sG25) bearing the motif sequence of 15 amino acids with the flanking sequences of 4 amino acids of g3p at the C-terminal end were prepared by use of a peptide synthesizer (9050 plus, Perseptive; Millipore, Bedford, MA). The lyophilized peptides of sC27 and sG25 were dissolved in a 0.5 M Tris-HCl buffer (pH 8.6) containing 4 M urea, then oxidized by bubbling air to form the intramolecular disulfide bond. The oxidation of the peptides was confirmed by reversed phase HPLC (μ-Bondasphere, 5 μ, C18-100A, 3.9 mm × 150 mm; Millipore Waters, Milford, MA). The oxidized form was eluted just in front of the reduced form on the chromatogram in both peptides. After bubbling for 24 h, the oxidized form was purified on the reversed phase HPLC. The monomeric form of the isolated peptide preparation was confirmed by MALDI-TOF mass spectrometry on Voyager DE-RP (Applied Biosystems, Foster City, CA). The peptide was lyophilized for storage. For use in cell culture experiments, sG25 (1 mg) was dissolved in 5 μl of dimethylformamide and diluted with phosphate-buffered saline (PBS; pH 7.3) to an appropriate concentration. The pH was adjusted with 1 M HEPES (pH 7.3; Dojin, Kumamoto). sC27 was dissolved in PBS. The amino acid sequences of the peptides were confirmed by use of a peptide sequencer (Model 473A; Applied Biosystems).

*Immunoblotting*—SDS-PAGE was performed as described (*1, 2*). Blotting was carried out with a semidry electroblotter (Sartorius, Tokyo) according to the manufacturer's instructions. After blocking with 5% skim milk, the PVDF membrane (Applied Biosystems) with blotted peptides was incubated with each anti-MCP-1 mAb at a dilution of 1:1,000 and alkaline phosphatase (AP)-labeled rat anti-mouse IgG at 1:1,000 (KPL, Gaithersburg, MD). The NTB and BCIP development system (Promega, Madison, WI) was used for the development.

*Panning of Phage Display Library*—A phage random display library was constructed, and micropanning was performed as previously described (*1*). The 15-amino acid sequence motif was inserted at the fifth position from the N-terminal of the g3p molecule in the fUSE5 vector. In this case, anti-MCP-1 mAb (24822.111 or F9) was used as a template to select phages. Briefly, phages ($1.2 \times 10^{12}$ transducing unit [TU]) expressing random peptide motifs were panned with an anti-MCP-1 mAb (24822.111 or F9; 10 μg)-coated 35-mm plastic plate (Iwaki Glass, Tokyo). The binding phages were eluted with a 0.1 N HCl-glycine buffer (pH 2.2). The eluate was immediately neutralized with 1.0 M Tris-HCl (pH 9.1) and amplified by infecting into starved K91kan cells. To select more specific phage clones, the amplified eluate was incubated with 5 μg of 24822.111 or F9 for the second round and 1 μg for the third round of panning at 4°C for 16 h. The reaction mixture was incubated with an anti-mouse IgG (5 μg)-coated 35-mm plastic plate at 4°C for 1 h to select specific binding phage clones.

*DNA Sequencing*—The DNA inserted in the phage clones was sequenced using a primer (5'-TGAATTTTCTGTATGAGG-3') by use of an ABI DNA sequencer 373A-36S, as described (*1*).

*Cell Preparations*—Human peripheral blood mononuclear cells (PBMCs) were obtained from healthy donors and isolated by the Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) technique. THP-1 (human monocytic cell line) was donated by Dr. Hideo Takeshima (Kumamoto University Medical School, Kumamoto) and maintained in an RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS).

*ELISA*—ELISA was performed as described (*1*). Briefly, microtiter plates (Nunc, Denmark) were coated with the monoclonal antibody (80 ng/40 μl) or the isotype-matched control antibody (mouse IgG1) and blocked with 1% BSA. Phage clones ($8 \times 10^9$ TU/40 μl) were added into wells and incubated with biotinylated anti-M13 mAb (40 ng/40 μl, Pharmacia, CA). The bound phages were detected by AP-conjugated streptavidin (Vector Laboratories, CA) in the presence of 1 mg/ml *p*-nitrophenylphosphate (Wako, Tokyo) with 10% diethanolamine (Wako). Absorbances were measured at 405 nm by use of a microplate reader (NJ-2300, Nunc, Tokyo).

*Flow Cytometry Analysis*—The experiment was performed as described (*1*). THP-1 cells ($5 \times 10^5$ cells/50 μl) were pre-incubated with human IgG (400 μg/ml) for 1 h to block Fc receptors. After washing cells with PBS containing 2% FBS and 0.1% $NaN_3$, they were incubated with or without 10 μg/ml of MCP-1, MIP-1α, or IL-8. After washing, the cells were incubated in the presence or absence of phage clones ($2 \times 10^{11}$ TU/ml) for 1 h. They were then incubated with 20 μg/ml of biotinylated anti-M13 mAb or 20 μg/ml of biotinylated anti-CCR2 mAb (R&D Systems) for 30 min, followed by the addition of fluorescein isothiocyanate (FITC)-conjugated streptavidin (Vecter, Buringama, CA). All reactions were carried out on ice, and analysis was performed using a Coulter EPICS XL (Coulter, Miami, FL).

*Chemotaxis Assay*—Monocyte chemoattractant activity toward human PBMCs was determined using a 48-well micro chemotaxis chamber (Neuroprove, Cabin John, MD) with a 5-μm pore size polycarbonate filter membrane as described (*17*). Briefly, MCP-1 (5 nM: 43 ng/ml) or IL-8 (5 nM: 40 ng/ml) was added into the lower chamber, while human PBMCs ($4 \times 10^4$ cells/40 μl/well) were added into the upper chamber with or without varying concentrations of antagonist (phage clones or peptides). The incubation time was 90 min for MCP-1 and 180 min for IL-8. After the incubation at 37°C in humidified air-5% $CO_2$, the filters were removed. Nonmigrating cells on the upside of the filters were wiped away; the filters were air-dried and stained with Diff-Quik. The migrating cells were counted under the microscope (magnification: 200). Data were calculated as % of control response induced with 5 nM MCP-1 or IL-8 alone. Each experiment was repeated at least three times.

## RESULTS

*Characterization of Anti-MCP-1 mAb*—We characterized two anti-MCP-1 mAbs, 24822.111 and F9. MCP-1 was treated with or without 2-mercaptoethanol (2ME) and heating and then electrophoresed on SDS-PAGE (15%). The gel was immunoblotted with each mAb as described in

"MATERIALS AND METHODS." The 24822.111 mAb reacted with both the non-treated and treated MCP-1, while F9 mAb bound to the non-treated preparation only (Fig. 1). These results suggested that 24822.111 recognized the primary structure, whereas F9 mainly reacted with the disulfide-linked conformational structure of the MCP-1 molecule. Both mAbs inhibited the MCP-1–induced chemotaxis of PBMC (87% inhibition by 24822.111, 44% inhibition by F9 mAb and 0% by control IgG at 3 μg/ml) in our experimental system.

*Selection of Phage Clones*—Panning of the phage display library was performed using the anti–MCP-1 mAbs. The isolated phage clones were tested for reactivity to each anti–MCP-1 mAb by ELISA (Fig. 2). When the 24822.111 mAb was used as a probe, we selected 25 clones which specifically bound to this mAb. In the case of F9, 42 clones were selected as specific binding phage clones. These clones showed no binding activities to isotype-matched control IgG1. The DNA sequencing of the g3p gene revealed three types of peptide motifs for each mAb (Fig. 2). The C27 or G25 was the clone exhibiting the strongest binding activity in the case of 24822.111 or F9, respectively. The 24822.111 mAb did not recognize G25, and F9 did not recognize C27. It is of note that two cysteines were found in all motifs, suggesting the important role of an intramolecular disulfide bond in these motifs. A homology search of all the motifs showed little similarity to the primary sequence of MCP-1. There is, however, a significant similarity among the selected motifs, particularly in the case of F9, whose consensus sequence was P(F)WYS(P)C at the side of COOH. The motifs selected by F9 may be related in the conformational structure of MCP-1. In the case of 24822.111, there is an ambiguous similarity between C27 and A3.

*Binding Activity of C27 and G25 Phage Clones to MCP-1 Receptors*—THP-1 cells express an MCP-1 receptor, CCR2 (Fig. 3A). Therefore, flow cytometry analysis was performed on THP-1 cells to determine whether the phage clones had the ability to bind to the MCP-1 receptors. As shown in Fig. 3, C and D, respectively, C27 and G25 exhibited remarkable binding activity to THP-1 cells, while a control phage clone L4 showed no binding activity (Fig. 3B). The binding activities of C27 and G25 were completely blocked by the preincubation of THP-1 cells with MCP-1, and partially blocked by preincubation with MIP-1α (Fig. 3, C and D). This result suggested that C27 and G25 bound to the other MIP-1α-receptor molecule expressed on THP-1. The preincubation of THP-1 cells with IL-8 showed no blocking effect (data not shown).

*Migration Inhibitory Activity of C27 and G25 Phage Clones on MCP-1–Induced Chemotaxis*—To examine the effects of C27 and G25 on the chemotactic activity of MCP-1, we assayed the migration of PBMC using 5 nM MCP-1 in the presence or absence of these phage clones (Fig. 4). Both C27 and G25 clones showed marked inhibitory activities at a similar level. The inhibition was observed at $1 \times 10^5$ TU/ml, and at $1 \times 10^8$ TU/ml it was almost complete in the case of G25 and 75% complete in the case of C27. The

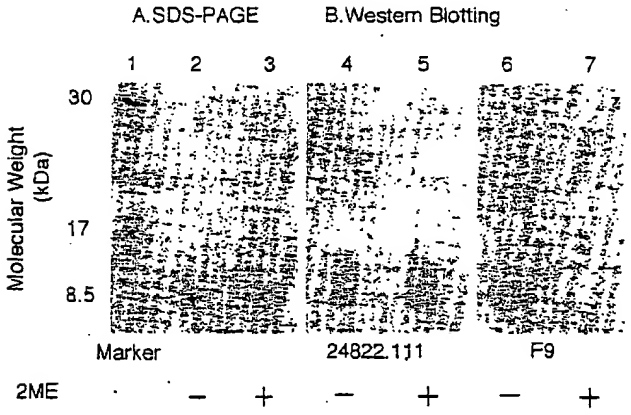


**Fig. 1. Characterization of anti–MCP-1 mAbs (24822.111 and F9).** (A) MCP-1 was treated with (+) or without (−) 2ME and electrophoresed on 10% SDS-PAGE. The gel was stained with Coomassie Brilliant Blue. The protein marker was in lane 1. (B) Immunoblot analysis of MCP-1 with 24822.111 and F9 mAbs under reducing (lanes 5 and 7) and non-reducing (lanes 4 and 6) conditions.

A

| anti-MCP-1 mAb | | motif | No./total |
|---|---|---|---|
| 24822.111 | C27 | RPLPPRFGCVPLGCL | 23/25 |
| | A3 | RGLVSCGLSVSCSLY | 1/25 |
| | B5 | TLVSMPGRPLQCRLC | 1/25 |
| F9 | G25 | NSGSICGFSVPWYSC | 40/42 |
| | H52 | TSLTGSFSCAPWYSC | 1/42 |
| | H2 | LQPHVFPGCSFWYRC | 1/42 |

B

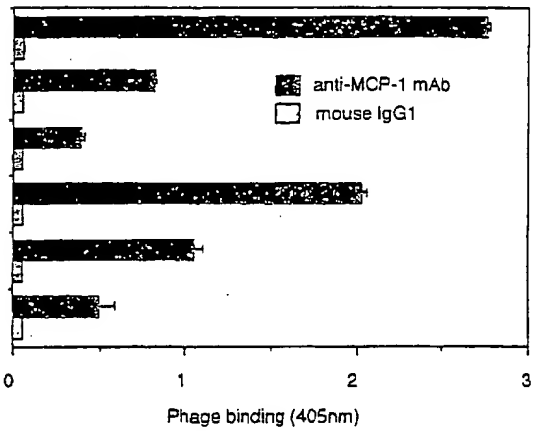


**Fig. 2. Phage clones selected by the anti–MCP-1 mAbs (24822.111 and F9).** (A) The motifs were determined by DNA sequencing. Three clones were selected by each mAb. Cysteines and the characteristic amino acid residues are shown by bold letters. (B) ELISA of phage clones recognized by anti–MCP-1 mAbs. The isotype-matched antibody was used as a control.

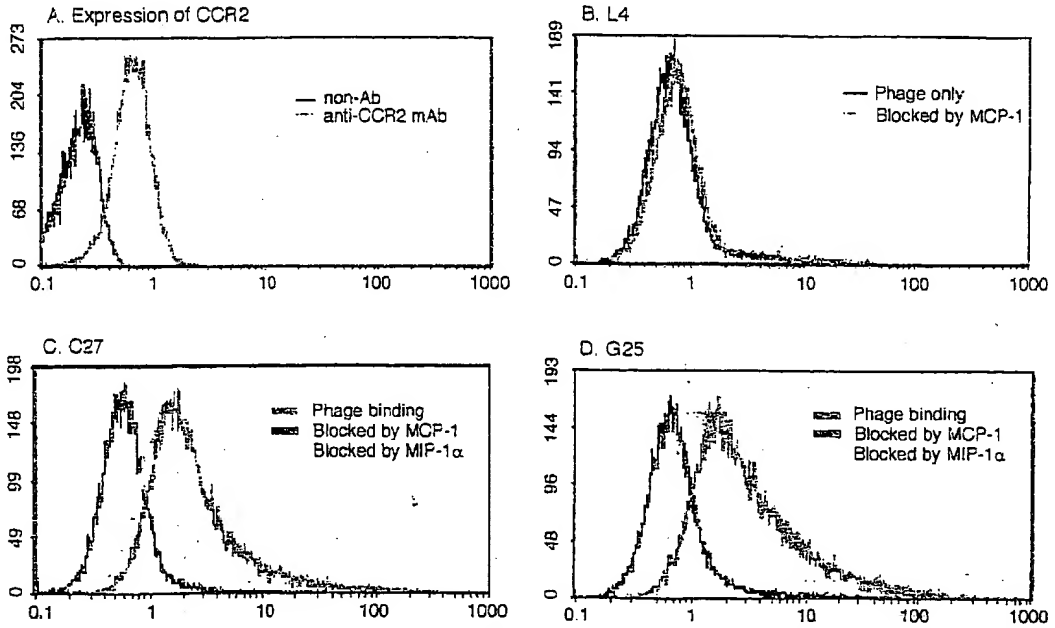


Fig. 3. **The binding of C27 or G25 phage clones to THP-1 cells on flow cytometer analysis.** THP-1 cells were preincubated with MCP-1, MIP-1α, or IL-8 (10 μg/ml). After washing, cells were incubated with or without phage clones ($2 \times 10^{11}$ TU/ml) for 1 h. The binding of phage was detected by the biotinylated anti-M13 mAb in combination with FITC-conjugated streptavidin. (A) Expression of CCR2 on THP-1. Cells were stained with anti-CCR2 (20 μg/mg). (B) No binding of a control phage clone, L4. (C, D) The binding of C27 or G25 to THP-1 cells preincubated with MCP-1, MIP-1α, or IL-8. The preincubation of IL-8 showed no effect on this binding (data not shown).

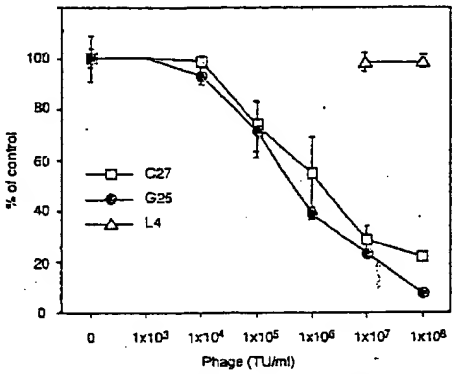


Fig. 4. **The C27 and G25 phage clones inhibit the MCP-1–induced chemotaxis of human monocytes.** The chemotaxis assay was performed as described in "MATERIALS AND METHODS." The representative results are presented as the mean ± SD value. The MCP-I–induced cell count without the inhibitor and the background count without MCP-1 and the inhibitor are 552 and 48 in this experiment. The control phage clone, L4, showed no inhibitory effect at $2 \times 10^8$ TU/ml.

control phage clone, L4, showed no inhibitory activity even at $2 \times 10^8$ TU/ml.

*Synthetic Peptides of the MCP-1–Binding Domain*—As C27 and G25 phage clones might display the motifs mimicking the MCP-1–binding domain, we chemically synthesized these peptide-motifs, sC27 and sG25. Since both motifs contain two cysteines in their sequences, we prepared control peptides by replacing cysteines by serines, which cannot form the loop structures. The preparations of sG25 or sC27 were intramolecularly crosslinked by a disulfide bond formed by air oxidation as described in "MATERIALS AND METHODS."

First, we tested the reactivity of these synthetic peptides with the anti–MCP-1 mAbs, 24822.111 and F9, by examining their inhibitory activity on the binding of the mAb to the C27 or G25 phage clone. sC27 inhibited the binding of the 24822.111 mAb to C27, while sG25 inhibited the binding of the F9 mAb to G25 (Fig. 5). The control peptides showed no blocking effects on these interactions. These results suggested that the disulfide formation of these peptides was critical for the binding to the anti–MCP-1 mAbs.

*The sC27 and sG25 Inhibit the MCP-1–Induced Chemotaxis*—As C27 and G25 phage clones inhibited the migration of PBMC induced by MCP-1, we examined the effects of sC27 and sG25 in the same assay system. sC27 and sG25 inhibited the migration in a dose-dependent fashion and showed 35 and 70% inhibition at a concentration of 1 mM, respectively (Fig. 6). On the other hand, the control peptides, sC27 Ser and sG25 Ser, had no effect (Fig. 6, B and C). These results suggested that the cysteines forming an intramolecular disulfide bond were necessary for binding to the MCP-1 receptors. sC27 and sG25 showed no influence on the chemotactic activity of IL-8. These results suggest that sC27 and sG25 may be MCP-1–binding domain mimics which specifically bind to the MCP-1 receptor.

## DISCUSSION

We have attempted to design functional peptide mimics by panning a phage display library using the anti-receptor/ligand mAb (*1–3*). We studied here the case of a chemokine, MCP-1, employing two anti–MCP-1 mAbs (24822.111 and F9) with distinct binding specificities (*17*). The 24822.111 mAb bound to both the 2-ME/heat–treated and the non-treated MCP-1 molecules, while the F9 mAb bound to only

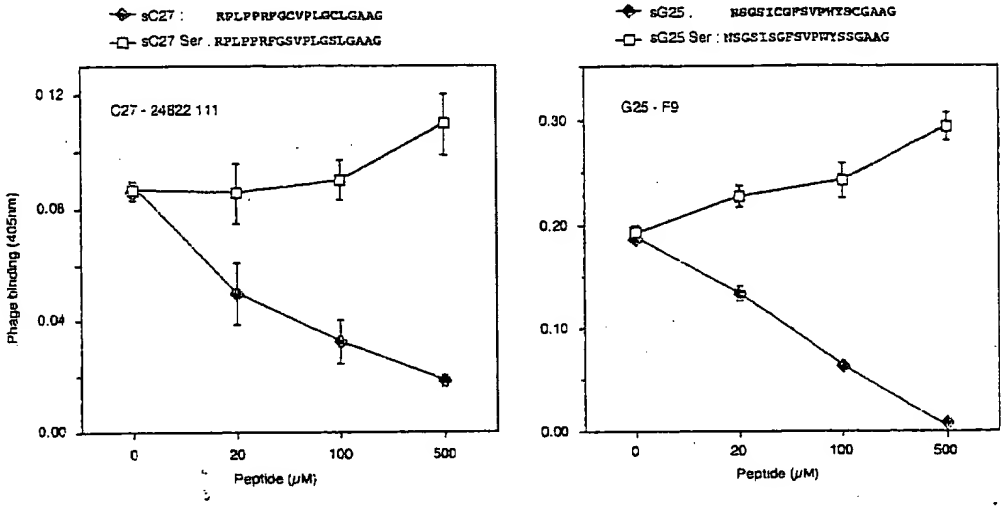


Fig. 5. **The synthetic motif-peptide inhibits the binding of anti-MCP-1 mAb to C27 or G25 phage clones.** The sC27 and sG25 peptides were chemically synthesized. The sC27 Ser or sG25 Ser peptide is a serine derivative in place of cysteine. The MCP-1 mAb (80 ng/40 μl/well: 24822.111 or F9) was coated in ELISA plates, followed by the addition of the C27 or G25 phage clones ($1 \times 10^8$ TU/50 μl/well) in the presence of varying concentrations of synthetic peptides. The concentration of phage clone was 1:100 lower than that used in the experiments of Fig. 2. The binding of phage clones was detected by biotinylated anti-M13 mAb in combination with AP-conjugated streptavidin. The chemically synthesized peptides (19mers) bear 15 amino acids of the unique motif sequence (bold letter) with the g3p-flanking sequence of 4 amino acids at the C-terminus.

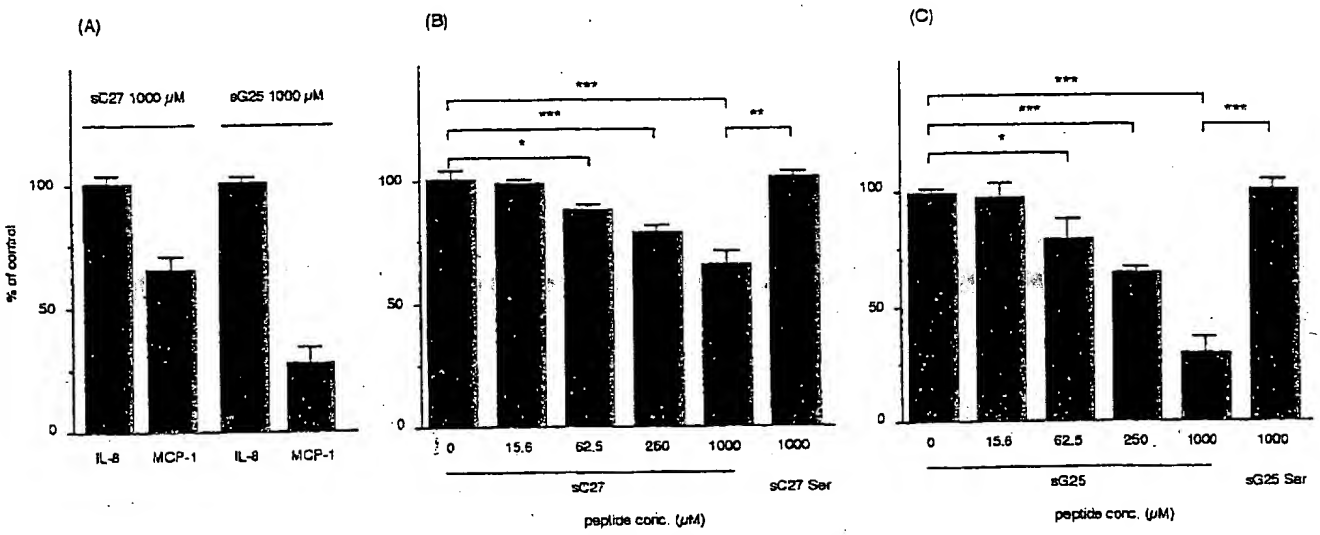


Fig. 6. **sC27 and sG25 inhibit the MCP-1–induced chemotaxis of human monocytes.** The migration of PBMC was induced with MCP-1 or IL-8 in the presence or absence of sC27, sG25, and their serine derivatives. The numbers of migrating cells are represented as % of control response induced with 5 nM MCP-1 (43 ng/ml) or IL-8 (40 ng/ml) alone. The experiments shown in this figure was performed in the same experiment. The migrating cell count with IL-8 was 275, and with MCP-1, 670; background response of cells alone was 56. The identical experiments were repeated three times. The representative results are presented as the mean ± SD. The significance of the difference was analyzed by ANOVA followed by post-hoc Fisher's PLSD test. A level of $p < 0.05$ was considered statistically significant. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

non-treated MCP-1 on the immunoblotting analysis (Fig. 1). From the fd phage display library, we isolated the C27 phage clone by probing with 24822.111 and the G25 phage clone by probing with the F9 mAb (Fig. 2). A phage displayed three to five copies of the motif-bearing g3p molecule (*1*). These phage clones showed not only binding activity to THP-1 cells, which was almost completely blocked by preincubation of the cells with MCP-1 (Fig. 3), but also migration-inhibitory activity on the MCP-1–induced chemotaxis (Fig. 4). These features suggested that the C27 and G25 phage clones might display the MCP-1–mimic motif on their g3p molecules. These results confirmed our previous experiments on CTLA4 and CCR5 (*1–3*). The prominent feature of this study is that we successfully synthesized the functional peptides sC27 and sG25 which inhibited the binding of the C27 and G25 phage clones to the corresponding mAbs (Fig. 5) as well as the chemotactic activity of MCP-1 (Fig. 6). However, a high concentration of these peptides was required for their activities, in comparison with the natural MCP-1. The binding affinity seems to decrease markedly when the motif is chemically synthesized. In agreement with these findings we failed to demonstrate the direct binding of sC27 or sG25 to THP-1 cells using biotinylated peptides by flow cytometry analysis (data not shown). This might have been the result of the steric hindrance caused by the biotinylation a

the N-terminal amino acid or the monovalent feature of the synthetic peptide in comparison with the multivalent display of g3p on the phage, in addition to the conformational alteration of the functional motif structure.

The sequence homology of the G25 and C27 motifs with MCP-1 was analyzed by using PIMA multiple-sequence-alignment software. The binding region of MCP-1 that interacts with its receptor, CCR2b, had been identified by mutation analysis (*18–23*). On searching for homology to this region (residue number 13–35), a sequence with very low similarity was found between a part of the C27 motif and the region of residues 24–29 of MCP-1 comprising the two characteristic arginine residues. It is uncertain why the C27 motif selected by the 24822.111 mAb did not reflect the primary sequences of MCP-1, as the mAb bound to the denatured MCP-1 preparation. The homology search of the G25 motif also showed little similarity to MCP-1.

It is reported that not only CCR2a/b but also CCR1, CCR4, CCR9, or CCR10 function as the MCP-1 receptor (*24, 25*). Regarding the binding-specificity of the C27 and G25 motifs, the preincubation of THP-1 cells with MCP-1 appeared to completely inhibit the binding of the C27 or G25 phage clone to THP-1 cells (Fig. 3). In contrast, MIP-1α (5 nM) exhibited partial inhibition on peptide binding (Fig. 3, C and D), while IL-8 showed no inhibition (data not shown). As receptor molecules for MIP-1α are reported to be CCR1, CCR4, and CCR5 (*24*), it is conceivable that both phage clones may bind to the structure of CCR2 shared with the other MCP-1 receptors. The precise binding specificity of these phage clones remains to be studied. When searching for a CTLA4-binding domain mimic (*1, 2*), we found an F2 motif which bound to CD80 but not CD86, whereas CTLA4 bound equally to CD80 and CD86. Thus, using this molecular-design approach, it is possible to find a serendipitous peptide molecule with binding specificity differing from the natural receptor/ligand-specificity.

In this study, we have not studied the Biacore analysis regarding the binding-affinity of the motifs to the MCP-1 receptors, because we have not conclusively specified the MCP-1 receptors as shown in Fig. 3, and because the purified phage g3p inevitably contained the aggregated form as described previously (*2*).

The results of Refs. *1–3* and this study together demonstrate the feasibility of copying the conformational image of the binding site, even though the binding specificity of the inhibitory mAb profoundly influenced the results. These C27 and G25 peptide sequences may be useful for the development of small molecules mimicking MCP-1.

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Schattauer

# Identification of Novel Peptide Antagonists for von Willebrand Factor Binding to the Platelet Glycoprotein Ib Receptor from a Phage Epitope Library

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## Summary

We have constructed a fusion phage epitope library in the filamentous bacteriophage *fuse5*. The library was made by inserting a degenerate oligonucleotide which encodes 15 variable amino acids into the $NH_2$-terminal region of the phage gene III protein. This library, containing over $10^7$ different epitope bearing phage, has been used in an attempt to identify inhibitors of the von Willebrand factor (vWF)-platelet Glycoprotein Ib interaction. The library was screened with a monoclonal antibody (RG46) that recognizes the GPIb binding domain of vWF (amino acids 445–733). A total of 30 clones falling into 8 classes have been identified that react with the RG46 antibody. Isolates from all 8 classes are positive by immunoblot analysis. The amino acid sequence of the gene III fusion protein from positive clones showed a strong homology to the known RG46 epitope. Peptides identified from the screen were synthesized and used to demonstrate that some of the synthetic peptides exhibited inhibitory activity towards ristocetin induced binding of vWF to the GPIb receptor. Thus, we have demonstrated that screening a fusion phage epitope library with a monoclonal antibody that inhibits vWF binding to the GPIb receptor can be a useful tool not only for mapping antibody recognizing determinants, but also can serve as a source for identifying novel peptides that are antagonists for vWF binding to the platelet GPIb receptor.

## Introduction

Ligand-receptor intervention is the basis for much of current pharmacologic research. In interactions where the ligand is a protein or peptide, the interaction usually involves only short regions (6–15 amino acid residues) of both the ligand and the receptor. The ability to screen large numbers of peptides for their potential agonist or antagonist effect could prove to be a source of pharmacologically useful compounds. Toward this end, numerous groups have constructed epitope libraries by either biological (1–5) or chemical (6–10) means. These libraries have proven to be useful for the rapid identification of epitopes to monoclonal antibodies (1, 2, 4–12) and have also been used to identify peptide sequences that bind to concanavalin A (13, 14) and to streptavidin (3). In principle it should be possible to use the same methodology to screen a library directly with a cellular receptor as the probe and to identify agonists or antagonists of the specific ligand/receptor interaction. However, there are only limited examples of direct library screening with probes other than monoclonal antibodies. One such example, although not unexpected, is the recent identification of RGD containing peptides that bind to either the GPIIb/IIIa receptor on platelets (15) or the $\alpha_5\beta_1$ integrin (16). Similarly, Smith et al. (17) screened a phage epitope library and identified peptides that bind S-protein, a 104 amino acid, inactive fragment of bovine pancreatic ribonuclease (RNase). When S-protein is combined with S-peptide, the enzymatic activity is restored. One of the peptides identified in the epitope library screen was shown to be an antagonist of the S-peptide/S-protein interaction, thus inhibiting the restoration of enzyme activity. This peptide was effectively regarded as a new RNase specific "drug". Other examples of screening directly with native proteins include the screening with the molecular chaperone BiP (18), as well as identification of phage that contain calmodulin regulatory peptides (19).

Given the relative ease of screening an epitope library with monoclonal antibodies, and the limited and somewhat specialized examples of screening directly with either native proteins or with cellular receptors, we elected to pursue screening of an epitope library with a monoclonal antibody that neutralizes the biological activity of a receptor/ligand interaction. For our initial screening we chose a well characterized antibody to von Willebrand factor (vWF) that disrupts vWF binding to the platelet GPIb receptor.

vWF is known to play a central role in the earliest stages of platelet deposition at the site of blood vessel wall injury (20–22). When the endothelial cell lining of a blood vessel is damaged, vWF is required for the subsequent adhesion of platelets to the subendothelium. vWF functions by binding to one or more components of the subendothelium including collagen (23, 24) and the heparin-like glycosaminoglycans (25). Binding of vWF to the GPIb receptor in turn triggers binding of fibrinogen to the platelet GPIIb/IIIa receptor and subsequent platelet aggregation (20, 21, 26). In pathological conditions, vWF may contribute to the processes that cause thrombotic vascular occlusion (27–30).

Previous studies have clarified some of the structural and functional characteristics of the vWF molecule, including the domain responsible for binding to the GPIb receptor (31–33). Specifically, experiments employing proteolytic fragments, synthetic peptides, and monoclonal antibodies have led to the demonstration that two discontinuous regions of vWF, spanning residues 474–488 and residues 694–708, are involved in binding of vWF to GPIb. Other studies have suggested that residues 514–542 may also contribute to GPIb binding (34). Similarly, residue 505 has also been implicated as being important in modulating vWF binding to the GPIb receptor (35). All of these studies have been hampered by the fact that, under low shear conditions, human vWF does not bind to GPIb in the absence of modulators such as ristocetin (36) or botrocetin (37).

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For our initial studies we chose to evaluate screening of the peptide epitope library by a murine monoclonal antibody designated RG46. This antibody was selected based on its ability to block the vWF-GPIb interaction by binding to a peptide sequence spanning residues 474–488 of the mature vWF subunit (31, 32). When this antibody was used in the present study to screen the epitope library, numerous positive clones were identified that reacted with the RG46 antibody. The predicted protein sequence of these phage closely resembled that of the known RG46 epitope. Synthetic peptides derived from these sequences not only inhibited antibody binding to vWF but also inhibited ristocetin-induced vWF binding to the GPIb receptor. These studies demonstrate that screening an epitope library with an antibody that neutralizes the vWF-GPIb interaction was able to identify novel peptide sequences that serve as antagonists for vWF binding to the GPIb receptor. These peptides have the potential for serving as "new lead" compounds for identification of novel antithrombotic agents.

## Experimental Procedures

### *Reagents and Peptides*

Enzymes and antibodies were purchased from New England Biolabs and Boehringer Mannheim respectively. The site directed mutagenesis kit was purchased from Amersham. DNA sequencing was performed using the Sequenase kit (United States Biochemicals) according to the manufacturer's protocol. Ristocetin was obtained from Biodata and avidin agarose was obtained from Pierce. Platelets were prepared as described by Marguerie et al. (38) and fixed with paraformaldehyde as described by Allain et al. (39). The *fuse5* vector and the bacterial strains K91 and K91Kan were kindly provided by Dr. George Smith. Electrocompetent *E. coli* DH10B cells were obtained from Bethesda Research Laboratories. The RG46 monoclonal antibody was a generous gift of Dr. Zaverio Ruggeri.

Purification of the RG46 antibody from ascites fluid was performed by a combination of $NH_2SO_4$ fractionation and affinity chromatography on a column containing a recombinant fragment of vWF encompassing amino acids 445–733 (40). Briefly, ascites fluid was adjusted to 50% $NH_2SO_4$, the precipitate collected by centrifugation, redissolved, and then dialyzed against three changes of phosphate buffered saline (PBS). Approximately 8 mg of antibody solution (1 ml) was applied to a 2 ml column containing 8 mg of the recombinant fragment coupled to agarose (AminoLink™, Pierce). Following binding, the column was thoroughly washed with PBS and the purified antibody eluted with glycine HCl at pH 2.5. Fractions containing the antibody were neutralized, pooled, dialyzed against PBS and then used for library screening or Western blot analysis.

Oligonucleotides were synthesized on an Applied Biosystems Model 380A DNA synthesizer. Peptides were either purchased from Cambridge Research Biochemicals Limited or were constructed manually using a custom built apparatus designed for the rapid simultaneous synthesis of 0.01–0.02 mmoles of peptide. Solid phase methodology using a 9-fluorenylmethyloxy carbonyl (FMOC) protection scheme in conjunction with the HOBT/Hbtu activation chemistry (41) was used. Peptides of interest were subsequently constructed in larger quantities (0.1–0.25 mmoles) using an Applied Biosystems Model 430 Peptide Synthesizer running Applied Biosystems Fast-moc® coupling cycle.

All peptides were cleaved for 1.5 hours at room temperature using a cleavage reagent of 82.5% trifluoroacetic acid, 5% phenol, 5% $H_2O$, 5% thioanisole, and 2.5% ethanedithiol (42). Following cleavage, the peptides were precipitated with ether, washed, then dried for 1 hour under vacuum. The peptides were then solubilized in either water, 10% acetic acid, or 10 mM ammonium bicarbonate depending upon the peptides net charge or solubility. Peptides were analyzed by reverse phase HPLC for purity and by ion spray mass spectrometry for molecular weight integrity. Mass spectrometry was carried out on a Sciex API III system in a solvent of 50% methanol and 5% acetic acid. A purity level of 95% was achieved for all peptides along with correct mass spectrometry data.

### *Construction of Vectors and the Peptide Epitope Library*

A control vector expressing the RG46 epitope an an $NH_2$ terminal fusion with the gene III protein was constructed from a pair of oligonucleotides that had SfiI cohesive termini and that encoded amino acids 474–488 of the mature vWF subunit (Figure 1). Oligonucleotides were phosphorylated with T4 polynucleotide kinase and then annealed with SfiI digested fuse 5 DNA followed by overnight ligation at 15° C. The ligation products were precipitated with ethanol, redissolved in water and then electroporated into either electrocompetent *E. coli* MC1061 or DH10B cells using a BTX model 600 electroporation apparatus. Electrotransformants were screened with an antisense RG46 oligonucleotide probe. Several hybridizing clones were isolated and their identity confirmed by DNA sequence analysis. The identity of a single isolate was further comfirmed by Western blot analysis of purified phage using the RG46 monoclonal antibody as probe. The data indicated a single immunoreactive band of the size expected from an RG46 epitope-Gene III fusion protein with no reactivity in an adjacent control lane containing non-recombinant phage (data not shown).

The *fuse5B* vector was constructed from the vector *fuse5* (5) by removal of an existing BstXI site in *fuse5* followed by replacement of the SfiI cloning sites with BstXI cloning sites. Site directed mutagenesis was carried out using the oligonucleotide described by Cwirla et al. (2).

For construction of the epitope library a collection of oligonucleotides encoding all possible 15mer peptides was synthesized. The sequence of the degenerate oligonucleotide as well as the half-site oligonucleotides was as described by Cwirla et al. (2) with the exception that $(NNK)_{15}$ was used instead of $(NNK)_6$ in the degenerate oligonucleotide.

The three oligonucleotides were phosphorylated with T4 polynucleotide kinase and then annealed with BstXI digested fuse 5B DNA followed by ligation and electroporation as described above.

### *Library Screening*

The peptide-epitope library was screened using a modification of the technique described by Parmley and Smith (43). Approximately $1 \times 10^{10}$ phage transducing units (tu) in 100 µl were incubated at 4° C overnight with the RG46 monoclonal antibody at a final antibody concentration of 67 nM. Biotinylated goat anti-mouse IgG (1 µg) was added and the mixture incubated for 1 h at 4° C, followed by the addition of 40 µl of a 50% aqueous slurry of avidin agarose and an additional 1 h of incubation. Unbound phage were removed by gently pelleting the slurry and discarding the liquid. The slurry was washed 3 times with gentle agitation in 1 ml of Tris buffered saline (TBS) containing 0.1% Tween-20 for 10 min at room temperature. The specifically bound phage were eluted with two separate additions of glycine HCl pH 2.2. Eluents were pooled and then neutralized with 2 M Tris base. The eluted phage samples were amplified by infecting exponentially growing *E. coli* K91-Kan cells, which were then plated on LB agar plates containing tetracycline (30 µg/ml) and kanamycin (30 µg/ml), and incubated overnight at 37° C. Phage from the amplified elutions were harvested from plates in TBS buffer, precipitated with polyethylene glycol, and then resuspended in 1 ml of TBS. One houndred µl of this amplified phage mixture was then used for the subsequent round of screening.

### *Colony Hybridization*

An aliquot of each elution was used to infect K91-Kan cells and the colonies transferred to Whatman 541 filters. Filters were processed sequentially through 0.5 N NaOH, 0.5 M Tris, pH 7.5, 2X SSC and 95% ethanol. Filters were prehybridized in 0.2% nonfat dry milk and 5X SSPE at 50° C for 2 h. Radiolabelled probe was then added and the filters hybridized overnight at 50° C. Filters were washed at room temperature twice in 5X SSPE + 0.1% SDS for 10 min, twice in 2X SSPE + 0.1% SDS for 20 min, and then at 50° C twice in 2X SSPE + 0.1% SDS for 20 min, and

twice in 0.5X SSPE + 0.1% SDS for 20 min. Filters were then exposed to x-ray film and positive colonies identified.

*ELISA Assays*

Immulon-4 microtiter trays were coated with a recombinant fragment of von Willebrand factor (40) at a concentration of 1 μg/ml for 1 h and then blocked with a solution of TBS containing 1% gelatin and 0.1% Tween. Peptides, at concentrations ranging from 1 nM to 1 mM, were preincubated with the RG 46 monoclonal antibody for 1 h before adding to the microtiter tray. The tray was incubated for 1 h and the extent of the reaction was monitored by addition of biotinylated goat anti-mouse IgG and streptavidin linked to alkaline phosphatase. The same second antibody preparation was used in both the library screening and the ELISA assays.

*Platelet Binding Assays*

The ability of the peptides to inhibit ristocetin-induced binding of vWF to platelets was carried out essentially as described previously (40). Fixed, washed platelets at a concentration of $10^8$/ml were incubated with $^{125}$I-vWF, ristocetin (800 μg/ml), and peptides for 1 h at room temperature. The $^{125}$I-vWF bound to platelets was separated from unbound vWF by pelleting through a sucrose cushion and the amount of bound vWF determined in a gamma counter.

## Results

A peptide epitope library containing random 15 amino acid sequences was constructed in a *fuse5* related vector. Transformation of this library into *E. coli* DH10B cells by electroporation yielded $5 \times 10^7$ independent transformants. Nucleotide sequence analysis of random clones from this library indicated that approximately 80% of the clones contained an inserted sequence.

As a first step we decided to evaluate the utility of the library for identifying peptides that inhibit the interaction of vWF with the platelet GPIb receptor. For this experiment we chose to construct a bacteriophage that encoded a peptide sequence that was known to inhibit ristocetin-induced binding of vWF to the GPIb receptor. This peptide sequence, comprising residues 474–488 of the mature vWF subunit, is also known to bind to a murine monoclonal antibody designated RG46 (31, 32). A pair of oligonucleotides encoding these amino acids, flanked by cohesive SfiI termini, were synthesized and inserted into the SfiI site of the vector fuse 5 in order to create a vector that encoded the known RG46 epitope as an $NH_2$-terminal fusion with the gene III protein (Fig. 1).

The control bacteriophage encoding the known RG46 epitope was then mixed with an aliquot of the unamplified peptide library at a dilution of $10^{-7}$ and the resulting library was screened with the RG46 antibody. Aliquots of bacteriopage obtained from elution at various cycles of the screen were amplified by infection and the resulting colonies were subjected to hybridization analysis with an oligonucleotide probe specific for the RG46 epitope encoding sequence. Table 1 indicates the total number of colonies obtained at each successive elution as well as those colonies containing a control phage encoding the RG46 sequence, as measured by colony hybridization. Following two rounds of affinity selection, a $10^5$-fold enrichment of this particular bacteriophage was observed. Similarly, after five cycles of affinity selection, this particular phage comprised 53% of the phage population representing an enrichment of $10^6$-fold over the initial $1 \times 10^{-7}$ dilution. This experiment strongly suggested that a specific epitope bearing fusion phage could be enriched from a large and diverse phage background.

Sixty clones from the fifth round of screening were then picked at random and subjected to DNA sequence analysis. The deduced amino acid sequence as well as the frequency of isolation of all the clones isolated in the screen is indicated in Table 2. As expected, thirty of these clones represented the control RG46 phage that had been seeded into the library. Eight clones were believed to be non-specific binding clones or second antibody binding clones since the inferred amino acid sequence did not resemble either the known RG46 epitope sequence or the sequence of any other phage isolated in the library screen (see the legend to Table 2). The protein sequence of the remaining phage bore a striking resemblance to the known RG46 epitope with a consensus sequence of PGGX followed by two hydrophobic residues (either L, I, or V) and an additional proline residue. The majority of these clones (17/22) also contained an acidic residue (D or E) following the PGG sequence, unlike the native RG46 epitope. In all cases where two or more clones had the same amino acid sequence, the nucleotide sequence encoding this peptide was identical.

As a first step in characterizing these peptide epitopes, synthetic 15 amino acid peptides were synthesized and used to compete for RG46 antibody binding to a fragment of vWF. For these studies we chose to use a recombinant fragment of vWF (RG12986) that contains amino

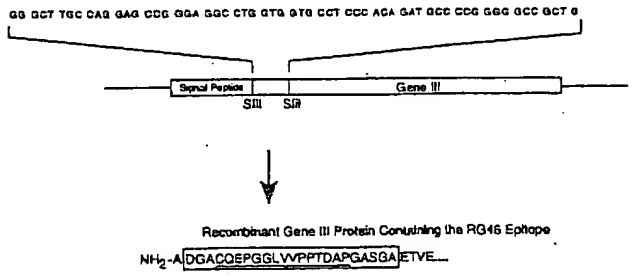


*Fig. 1* Construction of a phage containing the epitope for the RG46 monoclonal antibody. Synthetic oligonucleotides encoding the known RG46 epitope were constructed and inserted into the SfiI site of the fuse 5 vector. The nucleotide sequence of only the coding strand oligonucleotide is shown. The structure of the resulting recombinant gene III fusion protein is shown, with the putative RG46 epitope sequence underlined

*Table 1* Enrichment of RG46 epitope-bearing fusion phage from an epitope library. Phage bearing the RG46 epitope at the $NH_2$ terminus of the gene III protein were added to the fusion phage library at a $10^{-7}$ dilution and then subsequently screened with affinity purified RG46. Biotin labeled 2nd antibody and streptavidin agarose were used as the capture reagents. The experiment was carried out through five rounds of affinity purification with amplification of bound phage between each round. Eluted phage from each round were analyzed in a hybridization experiment. An oligonucleotide containing the DNA sequence coding for the RG46 epitope was hybridized to infected colonies at high stringency. The number of hybridizing colonies was compared to the total number of colonies in order to arrive at an enrichment value. TTU represents Tetracycline Transducing Unit

| Round | Total TTU | RG46 TTU | %RG46 TTU | Fold enrichment |
|---|---|---|---|---|
| 1 | 593 | 0 | – | – |
| 2 | 652 | 16 | 2.5 | $>10^5$ |
| 3 | 321 | 241 | 75 | $>10^6$ |
| 4 | 526 | 359 | 68 | $>10^6$ |
| 5 | 444 | 237 | 53 | $>10^6$ |

*Table 2* Amino acid sequence of epitopes. The amino acid sequence of the epitopes from the RG46 epitope library screen are aligned with the known RG46 epitope on von Willebrand factor. The frequency with which the isolates were identified in the screen is also indicated. The eight isolates (13%) which did not contain a consensus region are denoted NC. Five of these eight isolates were not specifically reactive when subjected to dot blot analysis with the RG46 antibody as the probe. The remaining three clones were believed to be specific for goat anti-mouse IgG as these clones reacted with second antibody alone in the dot blot assay (data not shown)

| Isolate | Frequency (%) | |
|---|---|---|
| RG46 | 50 | C Q E P G G L V V P P T D A P |
| G | 12 | F H H P G G D V I P L G I Q W |
| F | 8 | F D M Y P G G D L I P M F E S |
| H | 5 | R V N A A P G N D I I P F V Y |
| J | 3 | A F S P G A R I I P L S P W N |
| E | 3 | R P P R A F Y A M G G E L I P |
| D | 2 | T E Q Y G G M V I P Y V E R D |
| I | 2 | Y T L Q T P A L P G G Y L I P |
| K | 2 | G K F A L S H L A G G A I V P |
| NC | 13 | |

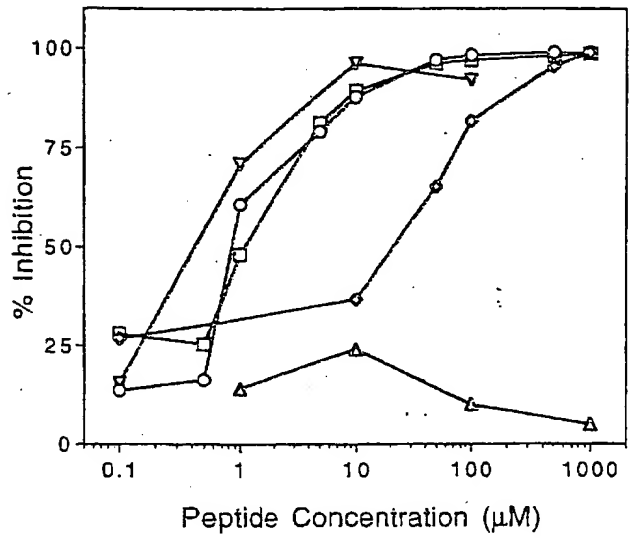


*Fig. 2* Inhibition of antibody binding to vWF by synthetic peptides. Microtiter wells were coated with a recombinant fragment of vWF (40, 44, 45). Increasing concentrations of either synthetic peptides or the vWF fragment were combined with monoclonal antibody RG46 and then added to the microtiter wells. The percent inhibition of antibody binding to the immobilized vWF fragment was measured as described in Materials and Methods. Data shown are for peptides derived from Clone E (◇), Clone F (○), the RG46 peptide (□), the negative control peptide (△), and the recombinant fragment of vWF (▽) as a positive control. The data are representative of two separate experiments. The sequence of the peptides tested are as follows:

| | |
|---|---|
| Clone E | RPPRAFYAMGGELIP |
| Clone F | FDMYPGGDLIPMFES |
| RG46 | CQEPGGLVVPPTDAP |
| Negative control | KPPTPPPEPET |
| Recombinant vWF fragment | vWF $Ser^{445}$-$Val^{733}$ |

acids 445–733 of native vWF (40, 44, 45). Peptides ranging in concentration from 1 nM to 1 mM were incubated with the RG46 monoclonal antibody and then added to the microtiter tray that had been previously coated with the vWF fragment. Following incubation the percentage of antibody binding to the vWF fragment was determined. Fig. 2 indicates typical inhibition curves for this assay. As expected, the RG12986 fragment positive control inhibited binding of the antibody to the immobilized RG12986 with an $IC_{50}$ of approximately 400 nM. Similarly, the synthetic 15 amino acid peptide based on the known RG46 epitope inhibited antibody binding to RG12986 with an $IC_{50}$ of 1 μM. Synthetic peptides based on the amino acid sequence of other phage isolated in the library screen were also tested in this assay and a summary of the $IC_{50}$ values obtained is indicated in Table 3. The $IC_{50}$ value obtained from all of these peptides were equal to or higher than the $IC_{50}$ of the known RG46 epitope based peptide. The $IC_{50}$ value of the peptide based on the sequence of clone F was approximately the same as the native epitope (Fig. 2) It is interesting that this clone was obtained at a relatively high frequency in the library screen, although in general there does not appear to be a correlation between the frequency of isolation and the $IC_{50}$ value in the antibody binding assay.

We also tested all of the above peptides for their ability to inhibit ristocetin-induced $^{125}$I-labelled vWF binding to the GPIb receptor. The data shown in Fig. 3 indicates typical inhibition curves for this assay. The $IC_{50}$ value obtained for the control RG46 peptide was approximately 350 μM which is in excellent agreement with previous reports for this peptide (34, 46). The RG12986 fragment was not tested in this system because of the previous observation that, in this assay, the RG12986 fragment paradoxically increases the apparent amount of vWF associated with the platelet pellet, possibly due to the formation of aggregates upon addition of ristocetin (40). The peptides derived from the sequence of clones J and F also inhibited binding of $^{125}$I-labelled vWF to the GPIb receptor. The sequence derived from clone F was nearly as effective as the peptide derived from the known RG46 epitope. As mentioned above, this clone was obtained at a relatively high frequency in the library screen and also has the lowest $IC_{50}$ in the antibody binding assay. However, in general, there does not appear to be a rigid correlation between the frequency of isolation, the $IC_{50}$ value

*Table 3* Analysis of synthetic peptides in an antibody binding assay and a platelet binding assay. Synthetic 15 amino acid peptides, based on the amino acid sequence of phage isolated from the RG46 library screen. were synthesized. These peptides were tested for their ability to bind to the RG46 antibody (Fig. 2 and Column A), and also for their ability to compete with vWF for binding to the GPIb receptor on platelets (Fig. 3 and Column B). The $IC_{50}$ values in the respective assays are shown and are the average of four separate experiments

| Isolate | | A $IC_{50}$ (μM) | B $IC_{50}$ (μM) |
|---|---|---|---|
| RG46 | C Q E P G G L V V P P T D A P | 1 | 350 |
| F | F D M Y P G G D L I P M F E S | 0.8 | 450 |
| D | T E Q Y G G M V I P Y V E R D | 2 | >1 mM |
| G | F H H P G G D V I P L G I Q W | 8.5 | >1 mM |
| H | R V N A A P G N D I I P F V Y | 10 | >1 mM |
| J | A F S P G A R I I P L S P W N | 18 | 600 |
| K | G K F A L S H L A G G A I V P | 18 | 850 |
| I | Y T L Q T P A L P G G Y L I P | 20 | 750 |
| E | R P P R A F Y A M G G E L I P | 20 | >1 mM |

in the antibody binding assay, and the $IC_{50}$ value in the ristocetin cofactor assay. A summary of the $IC_{50}$ values obtained for all of the peptides obtained in the epitope library screen is indicated in Table 3.

## Discussion

Binding of vWF to the GPIb receptor plays an essential role in mediating platelet adhesion and aggregation at the site of vessel wall injury. The interaction between these two is extremely complex in that vWF also interacts with other components in the vessel wall, including collagen and the heparin-like glycosaminoglycans. Further, binding of vWF to the GPIb receptor does not occur in vitro, under low shear conditions, unless nonphysiologic modulators such as botrocetin, ristocetin or other polycations are present.

Numerous studies have attempted to identify structural domains within vWF that are responsible for binding to the GPIb receptor [for a review see (47)]. In an extensive survey of synthetic peptides spanning vWF residues 449–728, Mohri et al. (32) suggested that two discontinuous regions of vWF comprising residues 474–488 and residues 694–708 are involved in ristocetin-modulated vWF binding to GPIb. Berndt et al. (34) suggested that peptides corresponding to these two regions are interfering with the modulator function of ristocetin rather than directly inhibiting vWF-GPIb interaction. Further, they provided evidence for complex formation between ristocetin and random peptides that contained proline repeats.

It was our goal to determine if an antibody that neutralizes the vWF GPIb interaction could be used to identify peptides, from a phage epitope library, that inhibit this ligand-receptor interaction. The RG46 antibody has been shown to react with residues 474–488 of the mature vWF subunit (32) and, as such, was expected to identify peptide sequences from the epitope library that resembled this sequence. The mechanism by which the 474–488 peptide inhibits vWF binding to the GPIb receptor is essentially irrelevant with regard to our main question of whether a neutralizing antibody can identify biologically active peptide sequences from the library.

In order to assess the feasibility of this approach, we initially constructed a phage that contained the precise RG46 epitope sequence and determined if this phage could be enriched from a diverse phage background. Indeed, a substantial enrichment of the phage was seen after only two rounds of screening and the phage had been selected to a nearly homogenous population by the third round of screening.

Having determined the feasibility of screening the library in a controlled manner, we next examined the inferred amino acid sequence of the remainder of the clones selected. Most of the clones identified in the screen revealed a strong homology to the known RG46 epitope and revealed a consensus sequence of PGGX followed by two hydrophobic residues (either L, I or V) and an additional proline residue. The majority of these clones (17/22) also contained an acidic residue (D or E) following the PGG sequence, unlike the native RG46 epitope. Berndt et al. (34) suggested that the vWF peptides from 474 to 488 (CQEPGGLVVPPTDAP) and 694–708 (LCDLAPEAPPPTLPP) shared significant structural features. In addition to being relatively proline rich, these peptides are also hydrophobic and are rich in aliphatic amino acids such as glycine, alanine, leucine, and valine. They also pointed out that 7 of the 10 proline residues are clustered as Pro-Pro or Pro-Pro-Pro. Of the peptides that were identified in the epitope library screen, all peptides contained at least one proline residue but none of the clones contained clustered prolines. It is also of interest that when more than one proline was present in the sequence, these proline residues were six amino acids apart and were separated by aliphatic amino acid residues.

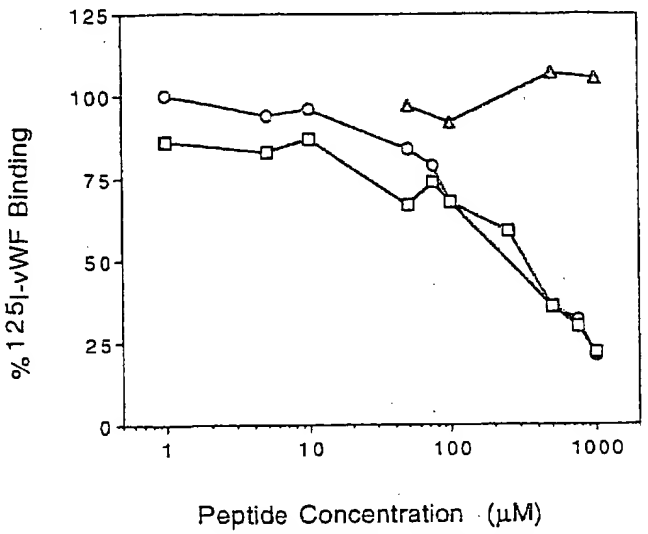


*Fig. 3* Inhibition of ristocetin-dependent binding of $^{125}$I-labeled vWF to fixed platelets. $^{125}$I-labeled vWF, platelets, peptides and ristocetin were combined and incubated for 60 min. The $^{125}$I-labeled vWF bound to the platelets was determined as described in Materials and Methods. Each data point is expressed as a % of $^{125}$I-labeled vWF binding to platelets in the absence of a competitor. Data shown are for peptides derived from Clone F (○), the RG46 peptide (□), and the negative control peptide (△). The sequence of the peptides are shown in the legend to Fig. 2. The data are representative of four separate experiments

Additionally, all peptides identified had amino acid sequence homology over a sequence of only seven residues. Four of these isolates had activity in the ristocetin cofactor assay, suggesting that if these peptides are inhibiting vWF binding to the GPIb receptor by interfering with the modulator function of ristocetin, then a peptide seven amino acids in length may be sufficient for interfering in this assay. The data also suggest that the epitope recognized by monoclonal antibody RG46 can be clearly assigned to the seven amino acid region described here.

It is unlikely that the peptides identified in the epitope library screen are capable of binding directly to platelets, although we have not examined this directly. Previous studies have suggested that the peptide from amino acids 474–488 did not inhibit botrocetin-induced vWF binding and platelet aggregation but did inhibit ristocetin and asialo-vWF-induced binding and aggregation (32, 46). Secondly, we have attempted, but failed to demonstrate (data not shown), direct binding of positive phage to platelets.

It is also interesting that none of these peptides exhibited a higher activity in the ristocetin cofactor assay than the native RG46 peptide. It is possible that all of the peptides selected are low affinity peptides due to multiple point attachment of the multivalent phage to the solid surface (2, 5, 48). This is one of the limitations of the fusion phage approach. Limiting the concentration of the antibody on the solid surface (49) or limiting the concentration of the gene III protein on the surface of the phage (48) have been suggested as alternative methods for obtaining high affinity peptides. Both of these approaches are currently being pursued.

In summary, we have demonstrated that screening a fusion phage epitope library with an antibody that neutralizes the vWF-GPIb interaction can serve as a source of novel peptides that are antagonists for vWF binding to the GPIb receptor. Through further structure/function analysis, these peptides have the capability to serve as "new lead" compounds for identification of potent antithrombotic agents.

*Acknowledgements*

We are grateful to Dr. George Smith for providing the vectors and strains for library construction and to Dr. Zaverio Ruggeri for providing the RG46 monoclonal antibody. We would also like to thank Dr. S. Tang and his colleagues for providing mass spectrometry data for the synthetic peptides and Dr. V. Chu for preparation of platelets.

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Received March 24, 1994 Accepted after resubmission September 13, 1994

| EXPRESS MAIL NO. EV462737349US | | |
|---|---|---|
| **DECLARATION OF ROSS STEIN UNDER 37 C.F.R. § 1.132**<br><br>Address to:<br>Commissioner for Patents<br>Alexandria, VA 22313-1450 | Attorney Docket<br>Confirmation No. | UCAL161DIV<br>7273 |
| | First Named Inventor | Steven Finkbeiner |
| | Application Number | 09/922,483 |
| | Filing Date | August 2, 2001 |
| | Group Art Unit | 1648 |
| | Examiner Name | U. Winkler |
| | Title | *Antibodies specific for proteins having polyglutamine expansions* |

Dear Sir:

1. I, Ross Stein declare and say I am an expert in the field of enzyme kinetics and drug discovery. I currently am affiliated with the Brigham and Women's Hospital in the Department of Neurology, the Laboratory for Drug Discovery in Neurodegeneration (LDDN). A copy of my curriculum vitae is provided herewith as Exhibit 2.

2. I understand that certain of the currently pending claims in the above-captioned patent application recite a screening method that assays the ability of a test agent to inhibit binding between an antibody specific for a polyglutamine expansion and a protein containing such a polyglutamine expansion. The screening method identifies agents that modulate the binding interaction between a protein comprising a polyglutamine expansion and a cellular target of such a protein.

3. I understand that a United States Patent and Trademark Office Examiner has rejected such claims on the basis that measuring the interaction between a polyglutamine expansion-containing protein and an antibody specific for a polyglutamine expansion will not provide any insight into the interaction of the polyglutamine expansion-containing protein and its cellular target.

4. In my opinion, it is reasonable to use such an assay to identify agents that inhibit binding of a protein containing a polyglutamine expansion to its cellular target. The antibody that will be used in this assay has a specific binding site for a toxic conformation of polyglutamine. It is not unreasonable to assume that the binding site on the antibody might have structural features and binding properties for polyglutamine that are similar to binding sites on the cellular proteins that mediate the toxic effects of polyglutamine. Therefore, small drug-like molecules that bind to the site on the antibody, thereby blocking polyglutamine from binding to the antibody, might also bind to these cellular proteins. I believe that this type of assay represents an important and valuable approach to identifying new therapeutic agents for neurodegenerative disorders.

5. I am principle investigator on a grant application submitted to the National Institute of Neurological Disorders and Stroke (NINDS) that includes a collaboration with Dr. Steven Finkbeiner and the use of as this assay as a key component. The grant application included the proposed use of such an assay in a high throughput screen format to identify agents that inhibit binding of a protein containing a polyglutamine expansion to its cellular target. This grant was reviewed be an NINDS Special Emphasis panel on April 23, 2004. The comments of the panel were strongly supportive of the science of the grant, including this assay.

6. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title XVIII of the United States Code, and that such will false statements may jeopardize the validity of the application or any patent issuing thereon.

7/14/04
Date

Ross Stein

## PART I: General Information

**Date Prepared: 03/10/2004**

**Name:** Ross Lee Stein, Ph.D.

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**Place of Birth:** St. Louis, MO

### Education

| Year | Degree | Institution |
|---|---|---|
| 1974 | B.S., Chemistry | Southern Illinois University<br>Edwardsville, IL |
| 1978 | Ph.D., Biochemistry | Indiana University<br>Bloomington, IN |

### Postdoctoral Training

| Year | Title | Place of Training | Specialty/Discipline |
|---|---|---|---|
| 1978 - 1979 | Postdoctoral Fellow | Washington University School of Medicine<br>St. Louis, MO | Enzymology/Pharmacology |
| 1979 - 1981 | Research Associate | University of Kansas<br>Lawrence, KS | Mechanistic Enzymology |

**Professional Positions**

| Year | Position/Title | Institution |
|---|---|---|
| 1981 – 1984 | Research Biochemist<br>Pharmacology Department | Stuart Pharmaceuticals<br>Wilmington, DE |
| 1984 – 1987 | Senior Research Biochemist<br>Pharmacology Department | Stuart Pharmaceuticals<br>Wilmington, DE |
| 1987 - 1989 | Associate Director<br>Department of Enzymology | Merck Research Labs<br>Rahway, NJ |
| 1989 - 1992 | Director<br>Department of Enzymology | Merck Research Labs<br>Rahway, NJ |
| 1992 - 1993 | Director<br>Enzymology | Genesis Pharmaceuticals<br>Cambridge, MA |
| 1993 - 1995 | Executive Director<br>Biochemistry | ProScript, Inc.<br>Cambridge, MA |
| 1995 - 1998 | Vice President<br>Biochemistry | ProScript, Inc.<br>Cambridge, MA |
| 1998 | Senior Director<br>Biochemistry | Cubist Pharmaceuticals<br>Cambridge, MA |
| 1999 – 2001 | Research Fellow<br>Chemical Enzymology | DuPont Pharmaceuticals<br>Wilmington, DE |
| 2001 - present | Director<br>Laboratory for Drug Discovery<br>in Neurodegeneration | Harvard Medical School<br>Boston, MA |
| 2003 - present | Associate Professor<br>Department of Neurology | Harvard Medical School<br>Boston, MA |

**Professional Societies**

| Year | Society | Role |
|---|---|---|
| 1972 - present | American Chemical Society | Member |
| 1976 - present | FASEB | Member |
| 1981 - present | AAAS | Member |

## PART II: Research, Teaching, and Clinical Contributions

### Summary of Research and Managerial Contributions

My research contributions have been in the areas of mechanistic enzymology and drug discovery. I have contributed to the understanding of mechanisms of catalysis of several enzymes, including: purine nucleoside phosphorylase, elastase, stromelysin, peptidyl prolyl *cis-trans* isomerase, the proteasome, ubiquitin C-terminal hydrolase, signal peptidase, γ-glutamyl transpeptidase, penicillin binding proteins, and aryl acylamidase. In all cases, I have applied precise kinetics-based probes to elucidate key structural features of catalytic transition states. The knowledge gained from these studies has been applied to the design and mechanistic characterization of inhibitors of these enzymes and, in several cases, to the development of new therapeutics for human disease. For elastase and the proteasome, these basic mechanistic studies contributed in a direct way to the development of a drug that was in clinical trials in the early 1990's for cystic fibrosis (i.e., elastase inhibitor) and a drug, VELCADE™, that has been approved by the FDA for the treatment of certain cancers (i.e., proteasome inhibitor).

As a manager, I have built and run research departments in both pharmaceutical and biotech companies. At Merck, I built the Department of Enzymology from seven scientists to about twenty five. This department contributed broadly to many projects at Merck that had enzymes as their therapeutic targets. Several of these projects resulted in drugs that went into human clinical trials. At ProScript, I was the company's first scientist and helped build the research department to about thirty scientists. As head of Biochemistry, I managed a staff that worked on the development of assays and elucidation of mechanism for enzymes of the ubiquitin-proteasome pathway. These enzymes include the proteasome, ubiquitin activating enzyme, ubiquitin conjugating enzymes, and ubiquitin C-terminal hydrolases. The work of this group of scientists was instrumental in the development of a proteasome inhibitor that is now in clinical trials.

### Current Research and Managerial Activities

My current activities as Director of the Laboratory for Drug Discovery in Neurodegeneration include hiring staff, managing the day-to-day operation of the facility, working with HMS staff and post-doctoral fellows to develop and optimize assays to be run as high-throughput screens, fund-raising activities, and conducting mechanistic studies on the compounds that are discoved in the various screens.

My research interests are in the areas of enzyme catalysis and inhibition. I am concerned with trying to understand the mechanistic origins of the enormous rate-accelerations that are effected by enzymes and how knowledge gained about enzymatic catalysis can be used to design inhibitors and understand how enzyme inhibitors work. Specific to these interests, I am currently undertaken two projects: acylation chemistry and dynamics of serine hydrolases and mechanisms of transglutaminase-catalyzed transamidation.

## Research Funding

"Harvard Center for Neurodegeneration and Repair Core D" 01/01/01 – 12/31/05
Principal Investigator: Peter T. Lansbury, Jr., Ph.D.
Agency: Harvard Medical School
The goals of Core D of the Harvard Center for Neurodegeneration and Repair are to discover new drugs for the treatment of neurodegneerative diseases and to discover tool-compounds that can be used to probe pathogenesis incell- and animal-models of disease. This project is being done in the Laboratory for Drug Discovery for Neurodegeneration, which was created as the means to best attain the overarching goals of Core D. The ongoing activities of the LDDN include the development and execution of novel cell-, enzyme-, and receptor-based assays for multiple disease targets. These assays are run against an in-house library of approximately 52,000 compounds. This project also includes medicinal chemistry-directed optimization of active compounds that are discovered during the screening process.

"Inhibition of antigen Presentation in Multiple Sclerosis" 12/1/02 – 11/30/06
Principal Investigator: Kai W. Wucherpfenning, M.D., Ph.D.
Agency: NIH
The specific aims of the project are: To develop robust assays for high-throughput screening of small molecules that block presentation of myelin peptides (Aim 1), to screen a large and diverse collection of small molecules under conditions relevant for intracellular peptide loading (Aim 2), and to study the mechanisms by which lead compounds inhibit presentation of myelin peptides (Aim 3). We are in a unique position to pursue this project, based on our combined expertise in immunology and drug discovery, and the preliminary data demonstrate that both assay development and high throughput screening are feasible.

"Chemical Genetics and Regulated Intramembrane Proteolysis" 7/1/03 – 6/30/08
Principal Investigator: Michael S. Wolfe, Ph.D.
Agency: NIH
The goal of this project is to apply the princiaples of chemical genetics to discover bio-organic agenst suitable of the studh of N and APP processing in signaling, developmental biology and human disease and to determine the protein targets of these agents.

"Metabolic Pathways and Defects in Fructose Metabolism" 7/1/03 – 6/30/08
Principal Investigator: Dean R. Tolan, Ph.D.
Agency: NIH
The major goals of this project are to 1) define sites for fructose assimilation and utilization using a combination of bioinformatics and molecular approaches, 2) determine a high-resolution structure of AP-aldolase, and use it to find stabilizing small-molecule ligands by both structure-based ligand design (SBLD) and using combinatorial chemistry, 3) create animal models for HFI using gene-targeting techniques, and 4) identify HFI mutations in the diverse US population, in particular Hispanic, African-American, and other ethnic groups that have not been well characterized, and correlate these findings to any specific phenotypes in these ethnic groups

"Compound Identification in Assays for Tau Pathology" 2/1/03 – 1/31/05
Principal Investigator: Kenneth S. Kosik, M.D.
Agency: NIH
Period: 2/1/03 – 1/31/05
The goal of this project is to develop tow high through-put screens aimed at the discovery of compounds with a potential impact on neurodegenerative pathologies related to tau

**Teaching Responsibilities**

My teaching responsibilities include formal and informal lectures, and the mentoring of post-doctoral students and technicians in the areas of enzyme kinetics, enzyme mechanism, drug discovery, and high throughput screening. Among the lectures are formal presentations at the Center for Neurologic Diseases (CND), Brigham and Women's Hospital and a lecture series on enzyme kinetics and mechanism given within the Laboratory for Drug Discovery in Neurodegeneration (LDDN). These lectures include the following:

"Role of Mechanistic Studies in Drug Discovery", CND, June, 2001.

"Principles of High-Throughput Screening", LDDN, September, 2001.

"Role of Mechanistic Studies in Drug Discovery – Assay Development", LDDN, October, 2001.

"Role of Mechanistic Studies in Drug Discovery – Kinetic Analysis of Enzyme Inhibitors", LDDN, December, 2001.

"Role of Mechanistic Studies in Drug Discovery – Principles of Elucidating Enzyme Mechanisms for Catalysis and Inhbition. I", LDDN, February, 2002.

"Role of Mechanistic Studies in Drug Discovery – Principles of Elucidating Enzyme Mechanisms for Catalysis and Inhbition. II", LDDN, May, 2002.

"Tranglutaminase as a Target for Huntington's Disease – Mechanism, Inhbitor Design, and High-Throughput Screenin", LDDN, February, 2003.

Mentoring has included numerous sessions with many post-docs, including:

Melissa Nicholson (Wucherpfennig lab, DFCI) – kinetics of receptor binding

Craig Justman (Lansbury lab, BWH) – mechanism of synuclein aggregation

Jae Ahn (Kosik lab, BWH) – kinase kinetics and screening for inhibitors

Malcolm Leissring (Selkoe lab, BWH) – kinetics of protease action

Yichin Liu (Lansbury lab, BWH) – kinetics and mechanism of ubiquitin hydrolases

David Wilson (Stein lab, LDDN) – kinetics and mechanism of tau aggregation

April Case (Stein lab, LDDN) – kinetics and mechanism of transglutaminase

**Teaching Experience – Invited Regional, National and International Contributions**

1. "Catalysis by Human Leukocyte Elastase." Presented to the Department of Chemistry, University of Kansas, July 1982.

2. "Catalysis by Human Leukocyte Elastase." Presented to the Department of Chemistry, Southern Illinois University, December 1982.

3. "An Automated System for Enzyme Kinetics." Presented at the First International Symposium on Laboratory Robotics, October 1983.

4. "Catalysis by Human Leukocyte Elastase." Presented to the Department of Chemistry, Georgia Institute of Technology, January 1984.

5. "Role of Subsite Interactions in Catalysis by Human Leukocyte Elastase." Presented to the Department of Pharmacology, Institute for Medical Research, Louis Pasteur University, Strasbourg, France, October, 1984.

6. "Role of Subsite Interactions in Catalysis by Human Leukocyte Elastase." Presented to the Department of Chemistry, Southern Illinois University, October, 1984.

7. "Mechanism of Action of Human Leukocyte Elastase." N.I.H. Workshop on Elastase Inhibition, June 10-11, 1985.

8. "Mechanism of Action of Human Leukocyte Elastase." Presented to the Department of Chemistry, University of Kansas, October1985.

9. "Mechanism of Action of Human Leukocyte Elastase." Presented to the Department of Chemistry, Wichita State University, October 1985.

10. "The Proton Inventory as a Probe of Protease Mechanism." Gordon Research Conference on Isotopes, February 1986.

11. "Mechanisms of Slow-Binding Inhibition of Serine Proteases." Presented to the Department of Chemistry, University of Kansas, November 1987.

12. "Mechanistic Insights into the Substrate Specificity of Human Leukocyte Elastase." Presented to the Department of Chemistry, University of Kansas (1987).

13. "Mechanisms of Slow-Binding Inhibition of Serine Proteases." Midwest American Chemical Society Meeting, Wichita State University, November 1987.

14. "Mechanisms of Slow-Binding Inhibition of Serine Proteases." Presented to the Department of Biochemistry, Tufts University, December 1987.

15. "Mechanisms of Slow-Binding Inhibition of Serine Proteases." Presented to the Department of Chemistry, University of Iowa, April 1988.

16. "Mechanistic Studies of Metalloproteases." Presented to the Department of Chemistry, Texas A&M University, March 1989.

17. "Mechanistic Studies of Metalloproteases." Presented to the Department of Biochemistry, University of Washington, April 1989.

18. "Mechanistic Studies of Peptidyl Prolyl cis-trans Isomerase." Presented to the Department of Chemistry, Florida State University, April 1990.

19. "Mechanistic Studies of Peptidyl Prolyl cis-trans Isomerase." Presented to the Medical School, Louis Pasteur University, Strasbourg, France, October 1990.

20. "Studies of the Matrix Metalloproteinase, Stromelysin." Presented to the Dept. of Pharmacy, Louis Pasteur University, Strasbourg, France, October 1990.

21. "The Proton Inventory as a Probe of Enzyme Mechanism." Presented to the Dept. of Pharmacy, Louis Pasteur University, Strasbourg, France, October 1990.

22. "Mechanistic Studies of Peptidyl Prolyl cis-trans Isomerase." Presented at Cyclosporin, Immunosuppression and Protein Folding, an international meeting held in Brandenburg, Germany, October 1990.

23. "Mechanistic Studies of Peptidyl Prolyl cis-trans Isomerase." Department of Biophysics, Boston University Medical School, November 1990.

24. "Mechanistic Studies of Peptidyl Prolyl cis-trans Isomerase." Department of Chemistry, Weslyan University, September 1990.

25. "Mechanistic Studies of Stromelysin Catalysis and Inhibition." International Conference on Cartilage Destruction and Repair, Redbank, NJ, September 1991.

26. "Solvent and Secondary Kinetic Isotope Effects as Probes of Protease Catlaysis and Inhibition." Gordon Conference on Isotope Effects in the Physical and Life Sciences, Ventura, CA, March 1992.

27. "Mechanistic Studies of Metalloproteinase Catalysis and Inhibition" Gordon Conference on Proteolytic Enzymes, Plymouth, N.H., June 1992

28. "Mechanistic Studies of Peptidyl Prolyl cis-trans Isomerase." Symposium on Enzymes, Inhibitors and Drug Design", Antwerp, Belgium, November 1992.

29. "Inhibition of Proteasome-Dependent Activation of NF-kB: A Novel Strategy for the Development of Anti-Inflammatory Agents", SRI Symposium: On the Cutting Edge of Anti-Inflammatory Drug Discovery", February 1996.

30. "Novel Inhibitors of the Proteasome", Gordon Conference on Proteolytic Enzymes, Plymouth, N.H., July 1996.

31. "Inhibition of Proteasome-Dependent Activation of NF-κB: A Novel Strategy for the Development of Anti-Inflammatory Agents", IBC's 2nd International Conference on Proteases Inhibitors, Washington, D.C. February 1997.

32. "Enzymology and Inhibition of the Ubiquitin-Proteasome Pathway", Gordon Research Conference on Enzymes, Coenzyme, and Metabolic Pathways, Kimball Union Academy, N.H., July, 1998.

33. "Kinetic and Mechanistic Studies of Signal Peptidase – A New Target for the Development of Antibacterial Agents", Temple University, School, of Medicine, Department of Biochemistry, May, 2000.

34. "Drug Discovery for Neurodegerative Diseases at Harvard Medical School", Meeting of the Parkinson Study Group, Santa Fe, NM, November, 2001.

35. "High-Throughput Screening and Drug Discovery for Neurodegenerative Diseases", Neurology Grand Rounds at Rhode Island Hospital, Brown University, School of Medicine, Department of Neurology, May, 2002.

36. "High-Throughput Screening and Drug Discovery for Neurodegenerative Diseases", Meeting of the Parkinson's Study Group, San Diego, CA, May, 2002.

37. "Ensemble of Transition States. Role of Conformational Mobility in Enzyme Catalysis", 29th Reaction Mechanisms Conference, Columbus, OH, June, 2002.

38. "New Models for Drug Discovery: Charting the Evolving Relationship between the Public and Private Sectors" Panel discussion at the IBC Drug Discovery Technology Conferency, Boston, MA, August, 2002.

39. "The Role of Mechanistic Studies in the Design of Enzyme Inhibitors," session organizer for national meeting of the American Chemical Society, Boston, MA, August, 2002.

40. "A New Model for Drug Discovery", Massachusetts Biotechnology Council's Conference on Biotechnology Investment, Boston, MA, October, 2002.

41. "A New Model for Drug Discovery", The Cure Parkinson's Project Conference – Accelerating the Cure, Chacago, IL, December, 2002.

42. "A New Model for Drug Discovery", Partners Program in Neurodegenerative Diseases 6th Annual Colluquim – Current Approaches to Understanding Neurodegenerative Diseases, Boston, MA, March 21, 2003.

43. "The Final Frontier – Therapies to Treat Neurological Disorders", session organizer, BIO 2003, Washington, DC, June 22-25, 2003.

44. "A New Model for Academic-Based Drug Discovery", BIO 2003, Washington, DC, June 22-25, 2003.

45. "Misguided Folding and Degradation of Protein – New Opportunities for Therapeutics in Neurodegenerative Diseases", BIO 2003, Washington, DC, June 22-25, 2003.

46. "A New Model for Academic-Based Drug Discovery", Drug Discovery Technology World Congress, Boston, MA, August 10-15, 2003.

47. "A New Model for Academic-Based Drug Discovery", Fourth Annual Institute for the Study of Agining Investiagotor's Meeting, Teaneck, NJ, October 1-3, 2003.

**Part III: Bibliography**

**Original Articles**

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**Thesis**

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